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Assays for ubiquitin-like protein ligation and proteasome function in archaea

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Abstract

Ubiquitin-like protein (Ubl) ligation is common to diverse archaea and targets many cellular pathways, including those associated with sulfur mobilization, and also tags proteins as substrates for degradation by the proteasome. Here we highlight protocols to assay proteasome function and Ubl ligation in archaea. A chase assay is described to monitor the impact of proteasome function on the stability of Ubl-modified proteins in the cell. A method to reconstitute Ubl ligation using a purified E1-like enzyme (UbaA), Ubl (SAMP2), methionine sulfoxide reductase A (MsrA), and cell lysate of an *msrA ubaA samp1–3* mutant is also described. MsrA is found to have the surprising ability to stimulate the formation of Ubl bonds. *Haloferax volcanii*, a halophilic archaeon originally isolated from the Dead Sea, serves as the model organism for these protocols.

1. Introduction

Archaea can target proteins for destruction by proteasomes using a ubiquitin-like protein (Ubl) ligation system (Fu et al., 2016; Humbard et al., 2010) (Fig. 1). Archaeal UbIs differ in primary sequence but share a common β -grasp fold and C-terminal diglycine motif that can be activated by E1-like enzymes (EC:2.7.7.) (Miranda et al., 2014, 2011). A Ubl~E1 thioester intermediate is formed that commits the Ubl to protein ligation instead of sulfur mobilization (Hepowit et al., 2016). Methionine sulfoxide reductase (MsrA) stimulates the Ubl ligation system by a mechanism that is independent of its methionine sulfoxide (MetO) reductase activity (Fu et al., 2017). The Ubl modification can transiently inactivate enzymes (e.g., Ubl-MoaE of molybdopterin biosynthesis) (Cao, Hepowit, & Maupin-Furlow, 2015) or target proteins (e.g., TATA-binding protein TBP2) for proteolysis by proteasomes (Fu et al., 2016). The archaeal proteasome system is composed of a network of 20S core particles (CPs) and AAA ATPases homologous to the eukaryotic Rpt1–6 proteasome subunits and Cdc48/p97 segregase (PAN and VAT, respectively) (Barthelme, Chen, Grabenstatter, Baker, & Sauer, 2014; Forouzan et al., 2012). The ATPases open the 20S CP gates into the CP interior and facilitate the unfolding and translocation of the target protein into the central proteolytic chamber of the 20S CP (Kim, Snoberger, Schupp, & Smith, 2015; Rabl et al., 2008). Associated with the archaeal Ubl-proteasome system are JAMM/MPN+

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metalloproteases of at least two distinct types (Hepowit et al., 2012). JAMM1 can cleave (iso) peptide bonds between the C-terminus of the Ubl and substrate and can reactivate enzymes such as Ubl-MoaE (Cao et al., 2015). By contrast, JAMM2 is inactive in vitro, most likely due to the absence of a key α -helix that is used by JAMM1 to bind the Ubl (Cao et al., 2017). This missing α -helix may be compensated by a protein partner(s), based on the finding that JAMM2 is needed in vivo for proteasome-mediated proteolysis (Fu et al., 2016). Thus, JAMM2 is proposed to release UbIs from target proteins prior to proteolysis.

Here we describe protocols for assays of proteasome function and Ubl ligation in archaea. *Haloferax volcanii* ATCC 29605, of the phylum *Euryarchaeota*, serves as the model system for these protocols as derivatives of this DS2 strain are amenable to genetics and protein biochemistry.

2. Methods for assay of proteasome function in archaea

To assay for proteasome function in degrading Ubl-tagged proteins, the abundance of a suspected target protein can be monitored by a chase assay that measures its rate of disappearance after shutoff of protein synthesis in proteasome mutant strains compared to wild-type cells. Chemical inhibitors can also be used to perturb proteasome activity. This section is based on Fu et al. (2016).

2.1 Monitoring the levels of proteasome targets in archaea

To monitor the levels of Ubl-modified proteins in *H. volcanii* for the chase assay, one can produce the target protein with an engineered C-terminal StrepII tag and the Ubl with an N-terminal Flag tag from an expression cassette. This approach frees the C-terminus of the Ubl to form (iso)peptide linkages with the target protein and frees the N-terminus of the target protein for possible regulation by the N-end rule, a mechanism of substrate targeting that requires a particular subset of N-terminal residues. The selection of the target protein is guided by previous detection of the protein in a Ubl-modified form. For this example, tandem mass spectrometry (LC-MS/MS) analysis revealed the TATA-binding protein TBP2 to be covalently linked to the Ubl SAMP2 by isopeptide bonds (i.e., Ubl linked to substrate lysine side chains) (Humbard et al., 2010).

TBP2-StrepII and Flag-SAMP2 are expressed on a bicistronic message from a gene cassette that uses an unregulated promoter (*P2rrnA*). Alternative promoters (Gregor & Pfeifer, 2005; Large et al., 2007) and epitope tags (Dilks, Gimenez, & Pohlschroder, 2005; Gimenez, Dilks, & Pohlschroder, 2007; Karadzic et al., 2012; Martin et al., 2018; McMillan, Hepowit, & Maupin-Furlow, 2016) are available for use in this halophilic archaeon as needed. A shuttle plasmid that includes selection markers and replication origins for *Escherichia coli* (ampicillin resistance and ColE1, respectively) and *H. volcanii* (novobiocin resistance and pHV2, respectively) is used to carry the cassette. The plasmid DNA is prepared from an *E. coli* *dam⁻ dcm⁻* strain that is deficient in DNA methylation, to ensure high transformation efficiency. During transformation, the *H. volcanii* cells are exposed to EDTA which strips off the S-layer of the cell wall, generating spheroplasts, and allows for polyethylene (PEG)-mediated uptake of the plasmid DNA into the cell (Dyall-Smith, 2009). The transformed cells are analyzed for steady state levels of TBP2 and Ubl SAMP2 by immunoblotting

analysis as described in Section 2.4. Cells carrying an empty vector are included as a control to ensure antibody specificity.

2.2 Generation of proteasome mutant strains

Genes predicted to impact proteasome function based on comparative genomics or other methods are deleted from the *H. volcanii* genome using a markerless deletion strategy (Cao et al., 2015; Fu et al., 2016; Miranda et al., 2011; Zhou, Kowalczyk, Humbard, Rohatgi, & Maupin-Furlow, 2008) (Fig. 2). The open reading frame (ORF) along with ~500bp of 5' and 3' flanking sequences is isolated from the *H. volcanii* H26 genome by PCR with primers that encode the desired restriction sites. The PCR product is ligated into the multiple cloning site of pTA131 (Allers, Ngo, Mevarech, & Lloyd, 2004) (see later) using restriction enzymes such as *Hind*III and *Bam*HI. The resulting “predeletion” plasmid is subsequently used as DNA template for inverse PCR to generate an in-frame deletion of the gene while avoiding disruption of adjacent ORFs.

The “deletion” plasmid is generated from this PCR product using KLD Enzyme Mix (NE Biolabs) that combines T4 polynucleotide kinase to phosphorylate the 5' ends of the PCR product, T4 DNA ligase to form circular DNA, and DpnI to cleave the methylated DNA template. The deletion plasmid is based on pTA131 (Allers et al., 2004), a vector that carries an origin of replication for *E. coli* (but not *H. volcanii*) and harbors *pyrE2*, a gene that codes for orotate phosphoribosyl transferase, an enzyme needed for uracil biosynthesis in *H. volcanii* H26 (*pyrE2*) (Bitan-Banin, Ortenberg, & Mevarech, 2003). The deletion plasmid is integrated into the *H. volcanii* genome by selecting for growth on casamino acid medium (CA) minus uracil (Dyall-Smith, 2009) at 42°C, within the optimal growth temperature range of 40–45°C reported for this archaeon (Mullakhanbhai & Larsen, 1975; Robinson et al., 2005). Isolated colonies are screened for plasmid integration by PCR using primers that anneal 500bp 5' and 3' to the target gene.

Subsequently, integrant-positive cells are subject to counterselection against the *pyrE2* gene in the integrated plasmid by inoculating into 4mL ATCC 974 medium supplemented with 5-fluoroorotic acid at 50µg mL⁻¹ (5-FOA medium) (last step in Fig. 2). Cultures are grown with rotary shaking in borosilicate glass tubes (13 × 100mm) at 42°C for 15h. Serial dilutions of the cultures are plated for isolating single colonies on 5-FOA medium; plates at dilutions with cleanly separated colonies are then patched onto the same 5-FOA medium. Clones of interest are identified by PCR with Taq or Phusion DNA polymerase using primers 700bp upstream and downstream, respectively, of the ORF. Mutant strains are further purified by restreaking on the same medium at least twice for isolated colonies. DNA sequencing of the PCR product is used to confirm the deletion.

If the gene appears to be essential for growth (i.e., no clones with the desired gene deletion are identified), a conditional allele can be generated (Large et al., 2007). The 20S proteasome β subunit gene is essential, for example (Zhou et al., 2008). For generating a conditional allele of the gene of interest, the native promoter in the *H. volcanii* chromosome is replaced with a tryptophan-inducible promoter (*Ptna4*) that can be repressed by removal of tryptophan from the medium (Large et al., 2007). For details on creating such a conditionally lethal strain, please see Large et al. (2007) and Zhou et al. (2008).

H. volcanii strains are stored short term (1–2 weeks) at room temperature in the dark (in plastic bags to maintain moisture) or long term in 20% (v/v) glycerol stocks at -80°C . *H. volcanii* strains are not stored at 4°C (haloarchaea are sensitive to cold shock). To generate the glycerol stocks, the strains are grown to stationary phase (OD_{600} 2.0–3.0) in ATCC974 medium and sterilely mixed 1:1 with 40% (v/v) glycerol. The stocks are transferred to -80°C . To initiate growth, the cells are inoculated by taking a small lump of frozen cells from the 20% (v/v) glycerol stock using a sterilized wooden stick or toothpick. The frozen cells are streaked for isolation from this toothpick onto ATCC974 medium supplemented with 2% (w/v) agar in a culture plate. Plates are transferred to zip-lock plastic bags with wet Kimwipes to prevent loss of moisture; the plates are incubated at 42°C until visible colonies form.

2.3 Assay of protein stability in proteasome mutant strains

To assess proteasome system function, the stability of proteins targeted for Ubl modification is assayed by chase assay in wild type and proteasomal system mutant strains. The TBP2-StrepII Flag-Ubl expression cassette described in Section 2.1 will be used as the example here. The expression plasmid is transformed into *H. volcanii* H26 (the parent strain used to generate mutations by homologous recombination) and mutant strains including in-frame deletions of the genes encoding the E1-like UbaA (Miranda et al., 2011), JAMM1/2 (Cao et al., 2015; Fu et al., 2016), Cdc48b/c (Fu et al., 2016), and PAN1/2 (Zhou et al., 2008) proteins. The cells are grown to log phase in 4mL ATCC 974 medium (an LB medium for salt-loving archaea) using $13 \times 100\text{mm}$ culture tubes with rotary shaking (200rpm). Log phase is determined based on an OD_{600} of 0.4–0.7 units using a UV–Vis spectrophotometer at 600nm. To bring the cells into the same exponential growth phase, the cells are diluted to an OD_{600} of 0.02 into 5.1mL ATCC 974 medium and again grown to log phase. The cells are centrifuged ($10,000 \times g$ for 6min at 25°C) and resuspended in 4.2mL of a buffered salt solution (4.1M NaCl, 150mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 140mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100mM KCl, and 20mM Tris–HCl, pH 7.5) to maintain cell integrity while removing the carbon/energy source.

The cells are treated with actinomycin D ($20\mu\text{g mL}^{-1}$) to inhibit DNA-primed RNA synthesis and anisomycin ($50\mu\text{g mL}^{-1}$) to block ribosomal-based protein synthesis. Stock solutions of the antibiotics are made by dissolving the actinomycin D at 10mg mL^{-1} in acetone and the anisomycin at 20mg mL^{-1} in methanol. The stock solutions are vortexed immediately prior to use and are added to the cultures to initiate the chase immediately following removal of cell aliquots to provide initial time points. The combination of these two inhibitors is found necessary to prevent the synthesis of new transcript and protein during the chase assay. Culture aliquots are removed at intervals between 0 and 2h and stored on ice prior to harvesting by centrifugation ($10,000 \times g$ for 6min at 4°C). Cell pellets are suspended in lysis buffer (50mM Tris–HCl, pH 6.8, 2M urea, 2% (wt/vol) SDS, and 0.1mg mL^{-1} EDTA-free protease inhibitor cocktail (Roche)) with glass beads (Chemglass Life Sciences). Cells are lysed with glass disruptor beads (0.1mm in diameter) by vortexing vigorously 5–7 times with cycles of 1min on and 1min off/on ice. Cell debris and glass beads are removed by centrifugation ($16,800 \times g$ for 20min at 4°C). Supernatants are transferred into fresh 1.5-mL microcentrifuge tubes on ice. The concentration of protein in the cell lysate is measured by

bicinchoninic acid (BCA) protein assay according to the supplier's instructions, using bovine serum albumin (BSA) as the protein standard (Thermo Scientific).

Cell lysate is mixed 1:1 with $2 \times$ SDS reducing sample buffer (100mM Tris-HCl, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 0.6mgmL^{-1} bromophenol blue, and 5% (vol/vol) β -mercaptoethanol) and boiled for 10min. Equivalent amounts of cell lysate (in the range of 5–20 μg per lane) for each time point are separated by SDS-PAGE (12%) in parallel gels. To assess equivalent protein loading, gel 1 is stained with Coomassie blue (Welinder & Ekblad, 2011). Proteins in gel 2 are analyzed by quantitative immunoblotting (see Section 2.4). In this way, the relative steady state levels (Fig. 3A) and the degradation rates (Fig. 3B–D) of the target protein in the various strains can be determined.

2.4 Immunoblotting analysis and quantification of Ubl-proteasome system targets

Proteins are electroblotted from the unstained SDS-PAGE gels onto a $0.45\mu\text{m}$ polyvinylidene difluoride (PVDF) membrane (Amersham) at 4°C for 3h at 90V or 13–14h at 30V using a mini trans-blot module according to the supplier's instructions (Bio-Rad). The membrane is placed upright in an $18 \times 10\text{cm}$ plastic container and briefly rinsed with 25mL of TBS (50mM Tris-Cl, pH 7.5, 150mM NaCl). The membrane is blocked for 2h at room temperature or overnight at 4°C in 25mL TBS supplemented with 0.05% (v/v) Tween 20 and 5% (w/v) nonfat dry milk. The primary antibody is added to 10mL of the blocking buffer in the following dilutions: 1:5000 of the Strep-tag II monoclonal antibody (in mouse) (QIAGEN, catalog number: 34850) to detect MsrA-StrepII or TBP2-StrepII; 1:10,000 of the HRP-conjugated His6 monoclonal antibody (Proteintech, catalog number: HRP-66005) to detect His6-UbaA; and 1:10,000 of the alkaline phosphatase-linked anti-Flag M2 monoclonal antibody (Sigma-Aldrich, catalog number: A9469–2MG) to detect Flag-His6-SAMP2. The membrane is incubated with the primary antibody solution for 1h at room temperature with medium speed rocking. The membrane is rinsed $3 \times 15\text{--}30\text{min}$ at room temperature with 25–50mL of TBS supplemented with 0.05% (v/v) Tween 20 (TBST) using the high setting of the rocker. A secondary antibody of goat antimouse IgG (whole molecule)-alkaline phosphatase-linked antibody (Sigma-Aldrich, catalog number: A5153–1ML) is used to detect the anti-Strep-tag II antibody. The secondary antibody is diluted 1:10,000 in 10mL of blocking solution and incubated with the membrane for 1h with medium rocking followed by rinsing of the membrane $5 \times$ in TBST buffer as described earlier. The antibody:protein complexes are visualized on the PVDF membrane by chemiluminescence using CDP-Star (Thermo Scientific) for the alkaline phosphatase-linked antibodies and ECL Prime (GE Healthcare) for the HRP-linked antibodies according to supplier's guidelines. The membranes are exposed to X-ray film (Thermo Fisher Scientific) for 1min for the anti-His antibody and for 10min for the anti-Flag and anti-Strep antibodies, with longer exposure times as needed. The intensities of the protein bands are quantified on the developed X-ray film using ImageJ (Schneider, Rasband, & Eliceiri, 2012). Future recommendations to enhance sensitivity in determining the turnover rate of the target protein are to label the proteome using ^{35}S methionine, inhibit protein synthesis, immunoprecipitate the target protein in time intervals, and monitor the level of radioactivity in the samples over time. A control strain devoid of the tag used to immunoprecipitate the target protein would be used to assess off-target signals.

3. Methods for reconstitution of archaeal ubiquitin-like protein ligation

3.1 Expression and purification of Ubl ligation system components

The E1-like enzyme UbaA mediates the ligation of Ubl to itself and MoaE (Hepowit et al., 2016) but requires additional factors, such as MsrA, to ligate the Ubl to other protein targets (Fu et al., 2017). To reconstitute Ubl ligation, the E1-like UbaA, Ubl SAMPs, and MsrA are purified (Fu et al., 2017; Hepowit et al., 2016). Cell lysate of *H. volcanii* mutant strains deficient in Ubl ligation is generated and used as a source of protein substrates (Fu et al., 2017). To maintain the stability of the *H. volcanii* proteins, 2M NaCl is included in the purification buffers, as this archaeon uses a “salt-in-cytoplasm” strategy for osmoregulation (Roesser & Muller, 2001). To cleave the Ubl from the target protein, the metalloprotease JAMM1 is purified and used as a control (Hepowit et al., 2012). Chromatography steps are done at room temperature as haloarchaeal proteins are typically thermotolerant. Samples are stored at 4°C. Protein concentration is determined by the BCA protein assay (Thermo Scientific) using BSA (Thermo Scientific) as a protein standard. Homogeneity of the proteins is determined by SDS-PAGE and mass spectrometry analysis (e.g., LC-MS/MS or ESI-TOF MS).

3.1.1 E1-like enzyme UbaA purification—The E1-like enzyme UbaA (HVO_0558) is expressed with an N-terminal His6-tag from the plasmid pJAM933 in *H. volcanii* NH02. To enhance the yield of His6-UbaA, NH02 is used since it is deficient in Ubl modification (*samp1/2/3*) and does not express UbaA from the genome (*ubaA*). The strain is grown to stationary phase (OD₆₀₀ of 3.0–4.0) at 42°C in ATCC974 medium with novobiocin (Nv, 0.2µg mL⁻¹) (4 × 1L in 2.8-L Fernbach flasks). Rotary shaking at 200rpm is used to aerate the cultures. The cells are harvested by centrifugation (4500 × g, 10min, 15°C). The cells are resuspended at 5mL g⁻¹ wet weight of cell pellet in Tris-salt buffer (2M NaCl, 50mM Tris–HCl, pH 7.4) with 40mM imidazole. Cells are lysed by 4–6 passages through a chilled French Press cell in a French Press at 20,000psi, with the lysate collected on ice. The lysate is clarified by centrifugation (12,000 × g, 20min, 4°C) and sequential filtration using 0.8 and 0.45µm pore size surfactant-free cellulose acetate (SFCA) filters (Nalgene). Sample is applied to a HisTrap HP Ni²⁺-affinity column (1mL bed volume, GE Healthcare) preequilibrated in Tris-salt buffer with 40mM imidazole and washed with 5–10 column volumes of the same buffer at a flow rate of 0.5mL min⁻¹. Proteins are eluted in Tris-salt buffer containing 500mM imidazole and pooled. Samples are dialyzed against Tris-salt buffer and concentrated to 0.5–2.0mg mL⁻¹ protein by centrifugal filtration using an Ultra-4 centrifugal filter unit with Ultracel-3 membrane (Merck) in an HL-8 swinging bucket rotor at 4°C and 4000 × g. Samples are further purified by gel filtration chromatography by applying the samples (0.5mL per run) to a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in Tris-salt buffer at 0.3mL per min. Protein samples purified by gel filtration are further concentrated to 10–15mg mL⁻¹ for future assay.

3.1.2 Ubl SAMP2 purification—The Ubl (SAMP2, HVO_0202) is ectopically expressed with an N-terminal Flag-His6-tag from the plasmid pJAM1132 in *E. coli* Rosetta (DE3). The *E. coli* strain is grown to log phase (OD₆₀₀ of 0.6–0.8) at 37°C in 25mL LB medium with kanamycin (Km, 50µg mL⁻¹) with rotary shaking at 220rpm. This culture

serves to inoculate 500mL LB+Km medium (in 2.8-L Fernbach flasks) to an OD₆₀₀ of 0.01; this larger culture is similarly grown to log phase. Isopropyl β -D-1-thiogalactopyranoside (IPTG, 0.4mM) is added to the culture to induce protein expression. The culture is shifted to 25°C and incubated for 12h with rotary shaking at 200rpm. The cells are harvested, and the protein is purified by Ni²⁺-affinity chromatography and gel filtration chromatography and concentrated, as described in Section 3.1.1.

3.1.3 MsrA purification—To prevent Ubl modification, the *H. volcanii* MsrA (HVO_A0230) is expressed in *E. coli* Rosetta (DE3) from plasmid pJAM3200. The MsrA is fused to a C-terminal StrepII tag to facilitate purification. The *E. coli* strain is used to produce MsrA-StrepII as described earlier for the Ubl SAMP2. The cells are resuspended at 5mLg⁻¹ wet weight of cell pellet in Tris-salt buffer (50mM Tris-Cl, pH 7.4, 2M NaCl) supplemented with 1mgmL⁻¹ EDTA-free protease inhibitor cocktail (Roche). The cells are lysed by French press, and the cell lysate is centrifuged and filtered, as described for the E1-like UbaA. The sample is applied to a StrepTrap HP column (5mL bed volume, GE Healthcare) equilibrated with Tris-salt buffer at a flow rate of 0.5mLmin⁻¹. The column is washed with 140mL Tris-salt buffer at a flow rate of 1.2mLmin⁻¹ to remove unbound proteins. Proteins are eluted in Tris-salt buffer supplemented with 5mM D-desthiobiotin at a flow rate of 0.5mLmin⁻¹. Fractions are pooled, dialyzed against Tris-salt buffer, concentrated, further purified by gel filtration chromatography, and concentrated, as described in Section 3.1.1.

3.1.4 JAMM1 metalloprotease purification—JAMM1 (HVO_2505) is ectopically expressed with an N-terminal His6-tag in *E. coli* Rosetta (DE3) from the plasmid pJAM991. Fresh transformants are grown at 37°C in LB medium supplemented with ampicillin (Amp, 0.1mgmL⁻¹) (0.5- to 1-L cultures) in 2.8-L Fernbach flasks with rotary shaking at 200rpm. At log phase (OD₆₀₀ of 0.4–0.6), IPTG (0.4mM) and ZnCl₂ (100 μ M) are added, and cultures are shifted to 25°C for 4.5–12h. Cells are harvested by centrifugation (10–15min at 9200 $\times$ g and 4°C) and washed in ice-chilled Tris-low salt buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl). Cells are stored at 80°C until lysis. Cells are resuspended in 5mLg⁻¹ wet weight of cell pellet in lysis buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 40mM imidazole, 1mM PMSF) and lysed thrice in a French Press (20,000psi). An equal volume of dilution buffer (20mM Tris-HCl, pH 7.5, 4M NaCl) is added to the cell lysate. The sample is clarified by centrifugation (twice for 10–20min at 9200 $\times$ g and 4°C) and filtration (SFCA filters, 0.2 μ m). Sample is applied to a HisTrap HP Ni²⁺ column preequilibrated in Tris-salt buffer with 40mM imidazole and washed with the same buffer at a flow rate of 0.5mLmin⁻¹. JAMM1 is eluted using Tris-salt buffer containing 500mM imidazole. Purified protein fractions are pooled and buffer-exchanged twice by dialysis into HEPES-salt buffer (20mM HEPES buffer, pH 7.5, 2M NaCl) for storage at 4°C as needed. Prior to assay, JAMM1 is purified by gel filtration chromatography using a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in Tris-salt buffer at 0.3mL per min. Peak fractions from the gel filtration chromatography step are then concentrated to 10–15mgmL⁻¹ by centrifugal filtration, as described previously.

3.1.5 Cell lysate preparation—Cell lysate is derived from *H. volcanii* LR03 (*samp1 samp2 samp3 msrA ubaA*). The *H. volcanii* strain is grown to stationary phase (OD₆₀₀ of 3.0–4.0) in ATCC974 medium supplemented with Nv and 25mMDMSO (0.5L in 2.8-L Fernbach flask) with rotary shaking at 200rpm. Cells are harvested by centrifugation (3500 × *g*, 10min at 15°C). Cell pellets are suspended in 8mL of Tris-salt buffer-7.5 supplemented with 10mMMgCl₂, 100μM bortezomib (LC Laboratories) and 30μgmL⁻¹ DNase I from bovine pancreas (Sigma). Cells are lysed in a French Press (20,000psi). Cell debris is removed by centrifugation (12,000 × *g*, 20min, 4°C). Cell lysate is filtered (0.2μm SFCA, Nalgene) and dialyzed 3 times against Tris-salt buffer at 4°C. As an example, 7mL of cell lysate is loaded into the dialysis tubing (3.5kDa MWCO, SnakeSkin prewet according to the manufacturer's instructions, Thermo Scientific). The sample (in the dialysis tubing) is placed in 4L of buffer with stirring for 1.5–2h at 4°C. Repeat this last step two more times.

3.2 Reconstitution of Ubl-protein ligation

Ubl ligation is reconstituted in 100μL reaction volumes distributed in 0.5mL Eppendorf tubes. The final components in the reactions are: cell lysate (80μL, 20–30mgmL⁻¹), 5μM MsrA-StrepII, 10μM Flag-His-SAMP2, 5μM His-UbaA, 50mM Tris-Cl, pH 7.5, 2mM MgCl₂, 4mM ATP, 25mMDMSO, 0.5mMDTT, and 2M NaCl. Components of the reaction are omitted to assess their impact. The reactions are incubated at 45°C for 0–18h. After assay, the salts are removed by application of the reactions to a Zeba Spin Desalting Column (7K MWCO) according to the supplier (Thermo Scientific). The desalted reaction products are separated by SDS-PAGE (12%) and analyzed by immunoblotting using anti-StrepII, anti-His6, and anti-Flag antibodies as described in Section 2.4 and shown in Fig. 4.

3.3 Cleavage of the Ubl modification

To cleave the Ubl from its target protein, HvJAMM1 (5μM) is added to Ubl-modified proteins (Fig. 5). The samples are incubated for 2–4h at 45–50°C. EDTA (50mM) is included to inactivate the metalloprotease as a negative control. HvJAMM1 can cleave Ubl-modified proteins that are generated in the cell (Fig. 5A) or are reconstituted in vitro (Fig. 5B). The cleaved products are desalted and analyzed by immunoblotting as in Section 3.2. To generate the Ubl-modified proteins in vivo to serve as substrates for cleavage by HvJAMM1, *H. volcanii* H26-pJAM949 expressing Flag-SAMP2 can be grown to stationary phase in 100mL of ATCC 974 medium supplemented with Nv (0.2μgmL⁻¹) and 100mM DMSO. The cells are harvested by centrifugation (15min at 9200 × *g* and 4°C), washed twice with 20mL of HEPES-salt buffer (20mM HEPES, pH7.5, 2M NaCl), and resuspended in the same buffer at 1g wet wt cells per 1mL. Cells are lysed by sonication (3 × 30–60s at 140W on ice). The lysate is clarified by centrifugation (as earlier) and its residual protease activity is inactivated by boiling for 20min. The boiled lysate is chilled, clarified by centrifugation (as earlier), and adjusted to 5mg protein per mL with HEPES-salt buffer. These samples containing Ubl-modified proteins formed in vivo can be stored at –80°C until use.

4. Conclusions

Assays for Ubl-protein ligation and proteasome function have proved fruitful in understanding how archaea may target proteins for energy-dependent proteolysis. The nanocompartmentalized structure of proteasomes and Ubl tagging make the Ubl-proteasome system energy dependent and provide a means to regulate the destructive forces of proteolysis. Finding that TBP2 may be phosphorylated at Ser2 prior to Ubl ligation provides an extra layer of regulation for substrate targeting in the archaeal cell. The reconstitution of Ubl ligation with E1-UbaA, SAMP2 and MsrA provides an opportunity to fractionate and identify the additional cofactors and/or substrates of the cell lysate that may be needed for this in vitro reaction. The recent development of SILAC (stable isotope labeling with amino acids in cell culture) for quantitative analysis of the *H. volcanii* proteome in a multiplex platform (McMillan et al., 2018) offers another avenue for discovery of novel factors associated with the Ubl-protein ligation system of archaea.

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Fig. 1.

Ubiquitin-like protein (Ubl) ligation and proteasome systems of Archaea. The Ubl protein (*blue*) is activated at its C-terminus by an E1-like enzyme (*red*). Adenylation (Ubl~AMP) and thioester (Ubl~E1) intermediates are formed. The Ubl is transferred from the Ubl~E1 to the target protein (*green*) by a mechanism that is stimulated by MsrA. The Ubl is linked by an amide (isopeptide) bond to the target protein. JAMM1 can cleave the Ubl-modified protein at the isopeptide linkage. The activity of JAMM2, AAA ATPases (Cdc48 and PAN), and 20S core particles is required for turnover of Ubl-modified proteins. X?, additional factors may be required.

Fig. 2.

Markerless deletion of a gene (*geneX*) predicted to impact proteasome function. A DNA fragment carrying *geneX* and 500bp 5' and 3' of the ORF is generated by PCR from *H. volcanii* genomic DNA. The DNA fragment is ligated into the multiple cloning site of pTA131. This "predeletion" plasmid is used as a template for inverse PCR (iPCR) using primers indicated by red arrows. The iPCR product is converted to the "deletion" plasmid using DNA polynucleotide kinase, T4 ligase, and DpnI (KLD). The deletion plasmid is transformed into an H26 *pyrE2* strain. Integrants, generated via homologous recombination, are selected by growth in the absence of uracil. The counterselective agent 5-fluoroorotic acid (5-FOA) is used to select for loss of the plasmid from the genome, which

can be resolved in the two ways shown. Wild type and mutant strains are distinguished at this stage by PCR screening.

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Fig. 3.

Proteasome system function evaluated in vivo by monitoring the stability of a protein target (TBP2) of the Ubl modification pathway. In this example, the E1-like UbaA, JAMM2, and the AAA ATPases (PAN2 and Cdc48c) are found to be important in the turnover of TBP2. TBP2 steady state levels (A) and turnover monitored by chase assay (B) in wt (wild type) and ubiquitin-like (Ubl) proteasome system mutant strains. Log-phase cells were treated with $20 \mu\text{g mL}^{-1}$ actinomycin D and $50 \mu\text{g mL}^{-1}$ anisomycin for the indicated times and collected for the chase assay. (C–D) TBP2 and SAMP2 intensities were quantified by ImageJ for the chase assay. Results are expressed as the percent change from time zero, which was set at 100%. The *H. volcanii* strains expressed TBP2-StrepII and Flag-SAMP2. TBP2 and SAMP2 abundance was determined by anti-StrepII and anti-Flag antibody

immunoblotting analysis (IB), respectively. Equal loading of total protein was confirmed by Coomassie blue (CB) staining. Data represent mean results $\pm$ standard errors of the mean (SEM) from three independent experiments. For details, see text and Fu et al. (2016).

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Fig. 4.

Reconstitution of ubiquitin-like protein ligation. Reaction components are MsrA-StrepII ($5\mu M$), Flag-His-SAMP2 ($10\mu M$), His-UbaA ($5\mu M$), cell lysate of LR03 ($H26\ msrA\ ubaA\ samp1-3$ mutant) ($80\mu L$, $20-30\text{mgmL}^{-1}$), 50mM Tris-Cl, pH 7.5, 2mM MgCl_2 , 4mM ATP, 25mM DMSO, 0.5mM DTT, and 2M NaCl. Components of the reaction are omitted to assess their impact. The time dependence of the reaction is demonstrated in lanes 7–11. In this example, the impact of MsrA on the reaction is examined by comparing lanes 4 and 6. For details, see text and Fu et al. (2017).

Fig. 5.

HvJAMM1-mediated cleavage of ubiquitin-like protein ligation products formed in vivo (A) and in vitro (B). (A) Reactions (10 μ L) included 5 μ g of heat-treated Flag-SAMP conjugate enrichment incubated for 2h at 50°C in assay buffer (20mM HEPES, pH7.5, 2M NaCl, 50 μ M ZnCl₂) with no additional effector (lane 1), 10 μ M HvJAMM1 (lane 2), or 10 μ M HvJAMM1 plus 50mM EDTA (lane 3). (B) Purified MsrA-StrepII (5 μ M) incubated in assay buffer (50mM Tris-Cl, pH 7.5, 2mM MgCl₂, 4mM ATP, 25mM DMSO, 0.5mM DTT, and 2M NaCl) with 1.6mgmL⁻¹ cell lysate of strain LR02 (H26 $\Delta msrA \Delta samp1/2/3$) expressing Flag-SAMP1 from plasmid pJAM947 for 18h at 45°C. The reactions were subsequently mixed with no effector (lane 1), 5 μ M HvJAMM1 (lane 2), or 10 μ M HvJAMM1 plus 50mM EDTA (lane 3). Proteins were separated by reducing 12% SDS-PAGE and analyzed by immunoblotting (IB: anti-Flag and anti-His) and total protein stain (SYPRO Ruby) as indicated on the *left*. Migration of molecular mass standards (*Mr*, kDa) indicated on *left*. Migration of SAMP2, SAMP2 conjugates, and HvJAMM1 indicated on *right*. The Ubl conjugates formed in vitro in Fig. 5B are less abundant than when reconstituted with purified SAMP and E1 (UbaA) (Fig. 4) but are highly dependent on MsrA and DMSO, as the level of E1 and its automodification are limited. For details, see text and Fu et al. (2017) and Hepowit et al. (2012).