

Specificity of archaeal caspase activity in the extreme halophile *Haloferax volcanii*

Mansha Seth-Pasricha,¹ Kelly A. Bidle² and Kay D. Bidle^{1*}

¹*Institute for Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ, USA.*

²*Department of Biology, Rider University, Lawrenceville, NJ, USA.*

Summary

Caspase-like proteases are key initiators and executioners of programmed cell death (PCD), which is initiated by environmental stimuli and manifests in organisms ranging from unicellular microbes to higher eukaryotes. *Archaea* had been absent from the caspase inheritance discussion due to a lack of gene homologues. We recently demonstrated extremely high, basal caspase-like catalytic activity in the model haloarchaeon, *Haloferax volcanii*, which was linked to the cellular stress response and was widespread among diverse *Archaea*. Here, we rigorously tested the catalytic specificity of the observed archaeal caspase-like activities using hydrolytic assays with a diverse suite of protease substrates and inhibitors compared with known model serine and cysteine proteases (trypsin, cathepsin, papain, and human caspase-8). Our experiments demonstrate that exponentially growing *H. volcanii* possesses a highly specific caspase-like activity that most closely resembles caspase-4, is preferentially inhibited by the pan-caspase inhibitor, zVAD-FMK, and has no cross-reactivity with other known protease families. Our findings firmly root the extremely high levels of caspase-like activity as the dominant proteolytic activity in this extreme haloarchaeon, thereby providing further support for housekeeping functions in *Haloarchaea*. Given the deep archaeal roots of eukaryotes, we suggest that this activity served as a foundation for stress pathways in higher organisms.

Introduction

Caspases are a family of highly refined, intracellular cysteine proteases that cleave a wide variety of substrate proteins at the C-terminus of an aspartate residue within specific tetrapeptide motifs. They generally display strong conservation in amino acid sequence, structure and substrate specificity (Cohen, 1997; Stennicke and Salvesen, 1998; Thornberry and Lazebnik, 1998) possessing a conserved domain structure with a histidine- and cysteine-containing catalytic diad. A variety of caspases have been identified in different metazoan animals, ranging from *Hydra* to humans (Thornberry and Lazebnik, 1998; Cikala *et al.*, 1999; Sanmartín *et al.*, 2005) that are key initiators and executioners of programmed cell death (PCD) or apoptosis, a genetically controlled, irreversible form of cell death that elicits specific morphological changes initiated by environmental stimuli (Lockshin and Williams, 1965; Kerr *et al.*, 1972).

Although PCD was first discovered in multicellular organisms, which use it for development and defence, it has now been shown to be a ubiquitous trait throughout nature, spanning diverse prokaryotes, and both unicellular and multicellular eukaryotes. PCD is catalysed either by classic caspases or orthologous proteins, such as metacaspases, paracaspases, phytaspases, and saspases (Vaux and Korsmeyer, 1999; Koonin and Aravind, 2002; Coffeen and Wolpert, 2004; Riedl and Shi, 2004; Salvesen and Abrams, 2004; Sanmartín *et al.*, 2005; Chichkova *et al.*, 2010; Mace *et al.*, 2010; Vartapetian *et al.*, 2011). Genomic, morphological and biochemical evidence of caspase-mediated PCD has also been documented in widely diverse evolutionary lineages of prokaryotic and eukaryotic phytoplankton including cyanobacteria, coccolithophores, diatoms, and dinoflagellates (Vardi *et al.*, 1999; Segovia *et al.*, 2003; Berman-Frank *et al.*, 2004; Bidle and Falkowski, 2004; Moharikar *et al.*, 2006; Bidle *et al.*, 2007; Bidle and Bender, 2008). These cumulative findings have peaked interest in exploring the molecular evolution of caspase-like proteins, their function, and the evolutionary drivers that have influenced their retention in different microbial lineages (Ameisen, 2002; Koonin and Aravind, 2002; Bidle and Falkowski, 2004).

Until recently, *Archaea* were absent from the discussion on the establishment, maintenance, and inheritance of apoptotic machinery, as their genomes lack clear homologues of these proteins (Koonin and Aravind, 2002).

Received 19 May, 2012; accepted 23 October, 2012. *For correspondence. E-mail bidle@marine.rutgers.edu; Tel. (+1) 848 932 3467; Fax (+1) 732 932 4083.

Re-use of this article is permitted in accordance with the Terms and Conditions set out at http://wileyonlinelibrary.com/onlineopen/OnlineOpen_Terms

However, the incidence and roles of caspase-like proteins are only now beginning to be recognized in this third domain of life. We recently demonstrated that the model haloarchaeon, *Haloferax volcanii*, exhibits very high, caspase-8-like (IETDase) activity and expression of immunoreactive proteins to human caspase-8 antisera, both of which were induced by salt stress and death and were abolished by *in vivo* addition of a broad-spectrum caspase inhibitor (Bidle *et al.*, 2010). Caspase inhibition severely impaired cell growth under low and high salt stress, suggesting a critical role in the cellular stress response (Bidle *et al.*, 2010). Furthermore, detection of similarly high catalytic activity and expression of immunoreactive proteins in other haloarchaea (*Halorubrum* and *Haloarcula*) and in diverse members of *Euryarchaeota* (the methanogen *Methanosarcina acetivorans* and the hyperthermophile *Pyrococcus furiosus*) and *Crenarchaeota* (the acidophile *Sulfolobus solfataricus*) argue for broad representation within the archaeal domain (Bidle *et al.*, 2010).

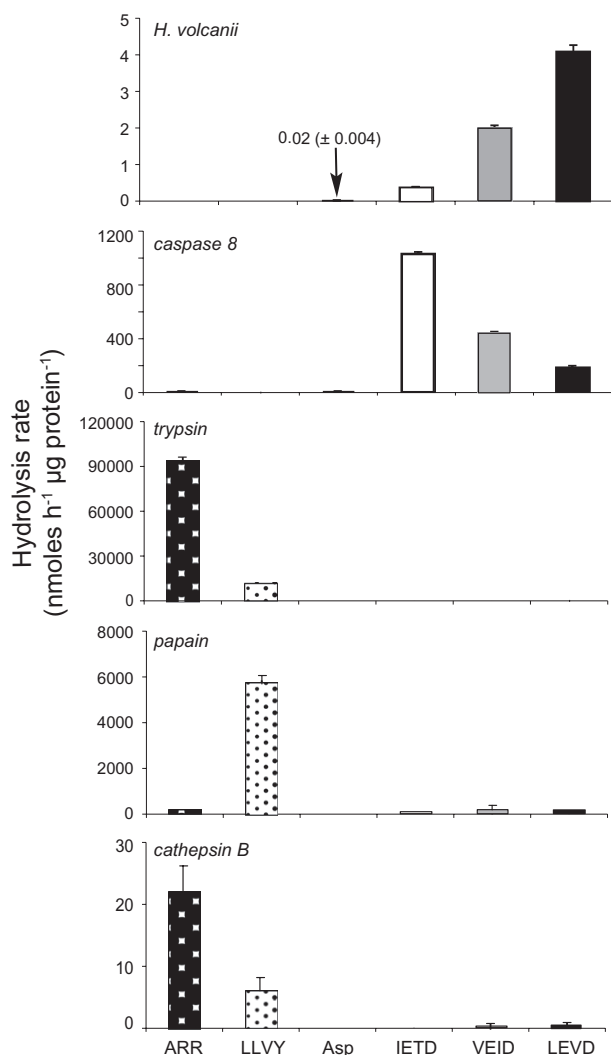
It is now evident that caspase-like proteins are likely to be present in all domains of life and are a widespread characteristic of unicellular organisms. By playing a role in normal cell function, caspase-like proteases in *Archaea* appear to participate in normal metabolic pathways, broadening their biological roles beyond apoptosis and cell death. Given the deep archaeal roots of eukaryotes (Yutin *et al.*, 2008), it is important to further explore the nature of this caspase-like activity in more detail in order to characterize its function and molecular evolution. It may represent a unique evolutionary lineage of these intriguing enzymes. Here, we present a detailed and focused characterization of substrate and inhibitor specificity for the observed caspase-like activities in *H. volcanii* so we can better understand the nature of these catalytic activities. We did not investigate the nature of the immunoreactive proteins to human caspase antibodies (Bidle *et al.*, 2010) in this study, since it is based on epitope similarities of denatured proteins and is not reflective of *in vivo* catalytic activities. Further, recent evidence demonstrates that even metacaspases, well-documented caspase orthologues (Uren *et al.*, 2000), have altered substrate specificities and are not responsible for observed caspase activities (Vercammen *et al.*, 2007). Hence, the respective catalytic and immunoreactive proteins in *H. volcanii* may not be justifiably linked and should be treated as distinct proteins until verified otherwise. Rather, we used a milieu of proteolytic substrates and inhibitors that target distinct classes of proteases, including specific caspases, to specifically determine if the observed caspase-like activity in *H. volcanii* was indeed caspase-specific or was a reflection of a more general manifestation of serine, cysteine, or metalloproteolytic activity. Our findings firmly root extremely high levels of specific caspase activity as the dominant proteolytic activity in this extreme halophilic archaeon.

Results and discussion

Specificity of caspase activity in H. volcanii cell extracts

We challenged cell extracts from exponentially growing *H. volcanii* cells with a diverse suite of canonical, fluorogenic caspase tetrapeptide substrates [YVAD- (caspase-1), VDVA- (caspase-2), DEVD- (caspase-3), LEVD- (caspase-4), WEHD- (caspase-5), VEID- (caspase-6), IETD- (caspase-8), and LEHD-AMC (caspase-9)] to expand our coverage from previously documented caspase-8-like activity (Bidle *et al.*, 2010) and to better elucidate the nature of caspase-like activity in this model haloarchaeon. *Haloferax volcanii* cell extracts displayed ~10- and ~fivefold higher specific activities with LEVD-AMC [74 232 relative fluorescence units (RFU) h⁻¹ mg protein⁻¹] and VEID-AMC (36 001 RFU h⁻¹ mg protein⁻¹) substrates, respectively, compared with IETD-AMC (6909 RFU h⁻¹ mg protein⁻¹). AMC fluorescence calibration curves placed the corresponding substrate hydrolysis rates at 4124, 2000, and 383 nmol h⁻¹ mg protein⁻¹ (Fig. 1). Much lower specific activities were observed for extracts incubating with the other caspase substrates; VDVA-, YVAD-, DEVD-, LEHD- and WEHD-based activities ranged from only 9% to 47% the IETD-based activity (data not shown). Notably, the same pattern of relative hydrolysis rates for LEVD-, VEID-, and IETD-AMC was observed in extracts from cells grown under low (1.5 M) and high (3.5 M) salt stress; VEID and IETD were 25–57% and 8–18% of these observed LEVD hydrolysis rates (Fig. 2). These findings may indicate the presence of either one or several proteins with overlapping activities and/or functions.

Our observed caspase-like-specific activities are by far the highest ever reported in a unicellular microbe, more notably in an exponentially growing archaeon. Reported caspase-specific (IETDase) activities in diverse unicellular marine phytoplankton, including cyanobacteria, diatoms, coccolithophores, and chlorophytes, are comparatively low for exponentially growing cells [10 s to 100 s of RFUs h⁻¹ mg protein⁻¹ (Segovia *et al.*, 2003; Berman-Frank *et al.*, 2004; Bidle and Falkowski, 2004; Bidle *et al.*, 2007; Bidle and Bender, 2008; Bidle and Kwitny, 2012)], with rates increasing ~10-fold when cells have activated autocatalytic cell death pathways. A wider survey of hydrolysis rates in unicellular microbes using other canonical caspase substrates has only been examined with the unicellular chlorophyte, *Dunaliella tertiolecta* (Segovia *et al.*, 2003), whereby hydrolytic cleavage of various caspase substrates was tested in cell extracts from stressed cells that were in different stages of light deprivation (0–5 days). Nonetheless, the maximum activity was seen for IETD and LEHD at ~300 RFU h⁻¹ mg protein⁻¹ after 5 days light deprivation and associated PCD activation. Overall, the activity rates for these



substrates ranged from 50 to 300 RFU $\text{h}^{-1} \text{mg protein}^{-1}$ over the 5 day time-course. Our hydrolysis rates for exponentially growing *H. volcanii* cells, along with our previous measurements in diverse *Archaea* (Bidle *et al.*, 2010), comparatively dwarf these rates from other unicellular microbes (by more than an order of magnitude), putting them in a unique catalytic and physiological context.

A comparison of substrate specificity for *H. volcanii* cell extracts to four model proteases, trypsin, cathepsin, papain, and recombinant human caspase-8, helped to further place it in the caspase catalytic class (Fig. 1). These model proteases represent distinct classes of peptidases that have well characterized active sites, substrate requirements, and inhibitors (Otto and Schirmeister, 1997; Hedstrom, 2002; Barrett and Rawlings, 2007; Vartapetian *et al.*, 2011). Not surprisingly, when challenged with a suite of fluorogenic substrates [ARR-AMC (trypsin and cathepsin), LLVY-AMC (papain), and IETD-AMC, VEID-AMC, and LEVD-AMC (caspase-8, caspase-6, and caspase-4

Fig. 1. Substrate specificity of *H. volcanii* cell extracts and various purified proteases. *Haloflex volcanii* strain DS70 (Wendoloski *et al.*, 2001) was grown aerobically at 45°C in an optimal 2.1 M salt medium (Bidle *et al.*, 2008). Cells were harvested from mid-exponential growth and cell extracts were prepared as previously described (Bidle *et al.*, 2010) with total protein concentration being determined using the BCA Assay (Thermo Scientific, Rockford, IL, USA). Substrate specificity assays were performed for *H. volcanii* cell extracts, recombinant human caspase-8 (22 ng; BioMol), trypsin (1 ng; Sigma), papain (10 ng; Fisher Scientific), and cathepsin B (250 ng; Sigma), with purified proteases verifying preferred substrate preferences. Cell extracts or purified proteases were incubated with a variety of fluorogenic substrates (final concentration of 100 μM ; Sigma): ARR-AMC, LLVY-AMC, Leu-AMC, Asp-AMC or canonical tetrapeptide caspase substrates (50 μM final concentration): IETD-AMC (caspase-8), VEID-AMC (caspase-6), LEVD-AMC (caspase-4; all from Enzo Life Sciences). Assays were performed in 1 \times Lauber Buffer (50 mM Hepes pH 7.4, 100 mM NaCl, 10% Sucrose, 0.1% CHAPS, 10 mM DTT). A modified Lauber buffer consisting of 1.5 M NaCl was used for *H. volcanii* cell extract assays (Bidle *et al.*, 2010). Kinetic analysis of substrate cleavage was performed (E_x 380 nm, E_m 460 nm) for 1 h at either 42°C (*H. volcanii* cell extracts only) or 37°C (all other proteases), with readings taken every 3 min using either a Spectra Max Gemini XS or a Spectra Max M3Plate Reader (both from Molecular Devices), using the SoftMax Pro 6.2.1 analysis program. AMC standard calibration curves were performed between 0 and 50 μM and were used to convert RFUs to nmol fluorogenic substrate cleaved. Cleavage rates for *H. volcanii* cell extracts and purified proteases (indicated in each panel) are reported as protein-normalized substrate hydrolysis rates ($\text{nmol h}^{-1} \mu\text{g protein}^{-1}$). Individual substrates tested are indicated on the x-axis and represented by different bar graph patterns. Error bars represent the standard deviation for triplicate measurements.

respectively)], each model protease had the highest activity with its preferred substrate (Fig. 1). Caspase-8 demonstrated notable selectivity among the caspase substrates, with highest catalytic activity for IETD-AMC. We did notice higher IETDase activity in the present study for purified human caspase-8 compared with what had been previously reported (Bidle *et al.*, 2010), likely due to a different source and batch of enzyme. *Haloflex volcanii* cell lysates had no measureable activity towards substrates of trypsin, cathepsin, and papain substrates under our incubation conditions at 42°C (Fig. 1).

We also tested for general leucine and aspartate aminopeptidase activity in *H. volcanii* extracts through hydrolysis of leucine-AMC (Leu-AMC) and aspartate-AMC (Asp-AMC), respectively, given the *H. volcanii* genome (<http://archaea.ucsc.edu/cgi-bin/hgGateway?db=haloVolc1>) has 13 annotated aminopeptidases (HVO_0242, *pepB* aminopeptidase II; HVO_0477, *ampS* aminopeptidase; HVO_0826, deblocking aminopeptidase; HVO_0836, aminopeptidase; HVO_1774, leucyl aminopeptidase; HVO_1829, aminopeptidase homologue; HVO_1849, aminopeptidase; HVO_2600, methionine aminopeptidase; HVO_A0535, aminopeptidase putative; HVO_1966, CAAX aminoterminal protease family; HVO_1997, CAAX aminoterminal protease; HVO_0082 CAAX amino terminal protease; HVO_0160, CAAX

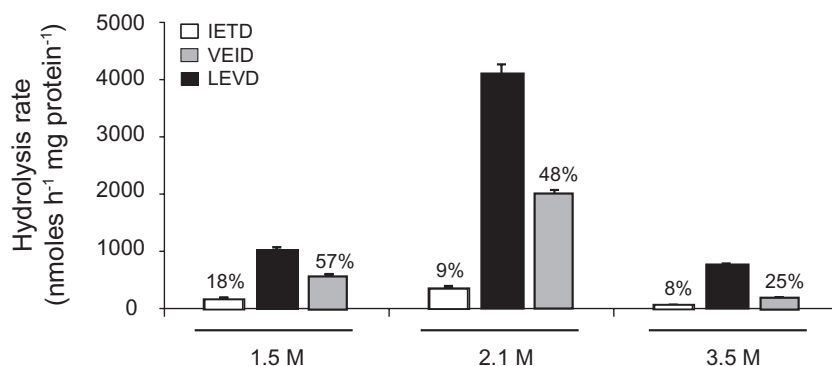


Fig. 2. Caspase-specific activities in cell extracts from *H. volcanii* cells grown in low (1.5 M), optimal (2.1 M), and high (3.5 M) NaCl concentrations and harvested at mid-exponential phase. Cell growth at respective salinities was performed as previously described (Bidle *et al.*, 2008). Extract preparation and kinetic cleavage assays were conducted as described in Fig. 1, with cell extracts incubating with LEVD-, VEID-, and IETD-AMC. Numbers above VEID and IETD bar graphs indicate the percent of LEVD activity. Error bars represent standard deviations for triplicate measurements.

amino terminal protease). Leu-AMC is a commonly used aminopeptidase substrate for diverse environmental microbes (Hoppe *et al.*, 2002) and has served as a model for general proteolytic activity. *Haloferax volcanii* grows at an acidic pH of ~5.1 and contains ~13 moles of Asp per 100 moles of total amino acids (Mullakhanbhai and Larsen, 1975; Hartman *et al.*, 2010), so Asp-AMC incubations helped verify that our observed caspase cleavage were specific to the C-terminal aspartate residue in tetrapeptide motifs, as opposed to a general aspartate cleavage response. Very low Asp-AMC hydrolysis activity (2 ± 0.4 nmol h⁻¹ mg protein⁻¹; Fig. 1) was detected in *H. volcanii* cell extracts, putting it at 0.05% LEVDase activity, while no detectable Leu-AMC hydrolysis was detected. For comparison, little to no activity was observed in *Escherichia coli* extracts incubated with Asp-AMC, but Leu-AMC displayed very high activity (2201 nmol h⁻¹ mg protein⁻¹).

Our results confirmed the consistent and extremely high basal activity in exponentially growing *H. volcanii* cells and refined the activity as more caspase-4-like, and demonstrate that the previous findings using IETD actually underestimated *H. volcanii* caspase activity. In order to address the possibility that the absence of some enzyme activities (e.g. LLVY-, ARR-, and Asp-AMC hydrolysis) in our extracts may have been due to competing small molecules, we also independently assayed for these activities in cell extracts heated to 60°C and in partially purified proteins at 42°C obtained using standard ammonium sulfate (AmSO₄) precipitation techniques (20%, 40%, 60%, 80% and 100%; Fig. S1; England and Seifer, 1990). *Haloferax volcanii* has two annotated serine proteases (Hv 1470: Putative intramembrane serine protease; Hv 2225: serine protease) and successful LLVY-AMC cleavage has only been reported at 60°C (Wilson *et al.*, 1999), a temperature that is considerably outside the organism's physiological, optimal growth temperature of 45°C (Robinson *et al.*, 2005) and more appropriate with temperature stress. Incubation of cell extracts at 60°C did yield LLVYase activity (228 ± 54 nmol h⁻¹ mg protein⁻¹); likewise, LLVYase activity was also observed in the 80%

and 100% AmSO₄ precipitate fractions, albeit much lower (5.7 ± 0.5 nmol h⁻¹ mg protein⁻¹; Fig. S1). Similarly low levels of Asp-AMC hydrolysis to those observed in cell extracts incubated at 42°C (Fig. 1) were measured both in 60°C heated extracts (16.6 ± 0.5 nmol h⁻¹ mg protein⁻¹) and in 80% and 100% AmSO₄ precipitate fractions (11.6 ± 0.2 nmol h⁻¹ mg protein⁻¹; Fig. S1). While heat treatment did not yield detectable ARR-AMC hydrolysis, relatively high activity was observed in the post-100% AmSO₄ soluble fractions (Fig. S1). Neither heat-treatment nor AmSO₄ precipitation recovered Leu-AMC hydrolytic activity. As with cell extracts, caspase activities were by far the highest activities recovered in partially purified protein fractions (Fig. S1); they were generally enriched in 80% and 100% AmSO₄ fractions and showed the same relative pattern of substrate hydrolysis (LEVDase > VEIDase > IETDase). Boiling of cell extracts at 100°C for 30 min completely abolished all hydrolysis activities, indicative of protein dependence. Our results confirm prior observations (Wilson *et al.*, 1999) and provide support for inhibitory molecules of LLVYase activity in cell extracts. Nonetheless, it was particularly striking that LLVYase activity in *H. volcanii* lysates represented only a very small fraction (< 5%) of the maximum observed caspase activity (i.e. LEVDase) and was not detectable at physiological temperatures.

Selectivity of caspase inhibition

The specificity of caspase-like activity exhibited in *H. volcanii* was further verified by challenging cell extracts with a panel of protease inhibitors that target a diverse array of proteases including serine proteases (phenylmethylsulfonyl fluoride, PMSF; aprotinin, Apr), cysteine proteases (leupeptin, Leu; E-64), metalloproteases (EDTA), and a cocktail of these inhibitors (pooled inhibitors, PI) or caspases (z-VAD-FMK). The aforementioned four model proteases, trypsin (serine protease), papain and cathepsin (cysteine proteases), and caspase-8 (caspase), were used to diagnose the specificity and efficacy of the chosen inhibitors when incubating with their preferred substrate.

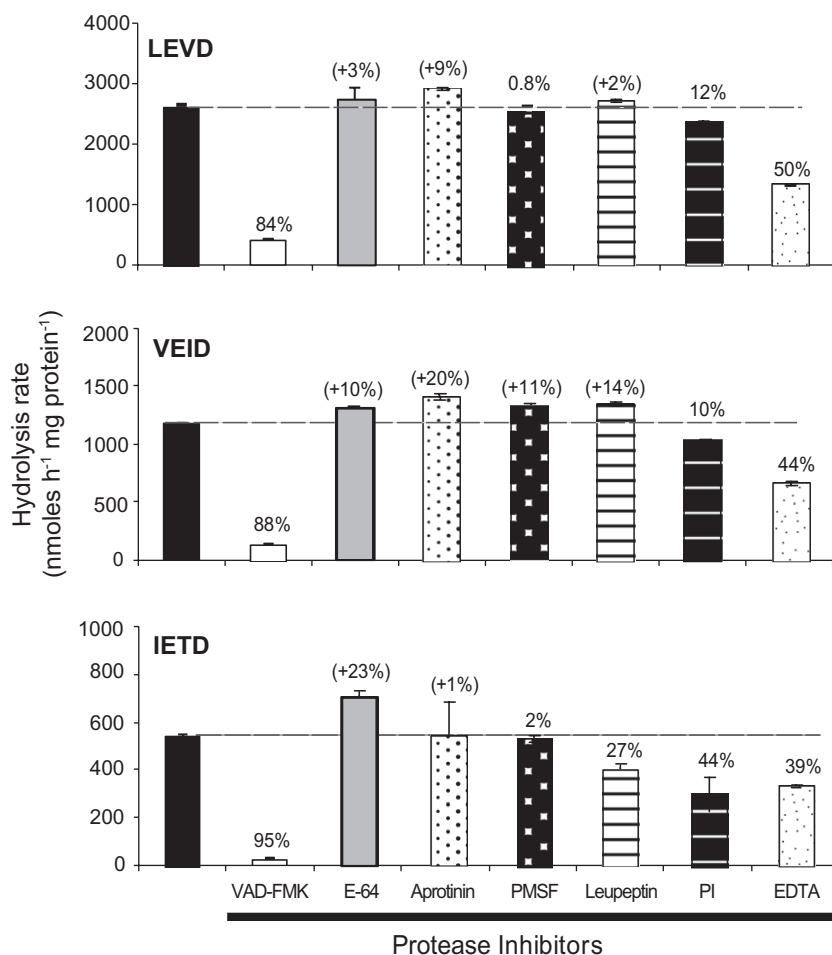


Fig. 3. Selective inhibition of caspase activity in *H. volcanii* cell extracts. The efficacy of various protease inhibitors was tested against the cleavage of canonical fluorogenic caspase tetrapeptide substrates (IETD-AMC, LEVD-AMC, and VEID-AMC) as another test of the specificity of activity in *H. volcanii* cell extracts. Cell growth, extract preparation, and kinetic cleavage assays were conducted as described in Fig. 1. The panel of protease inhibitors (10 μ M final concentration) were added individually to each reaction included: phenylmethylsulfonylfluoride (PMSF), aprotinin, leupeptin, E-64, protease inhibitor 'cocktail' (PI), and EDTA (all from Sigma), as well as the pan-caspase inhibitor zVAD-FMK (20 μ M final concentration; Enzo Life Sciences) as previously described (Bidle *et al.*, 2010). Fluorogenic substrates were added to cell extracts after a 1 h pre-incubation at 42°C with each protease inhibitor treatment and the degree of inhibition (%) was normalized to the measured activities in uninhibited control reactions. The degree of inhibition (%) was normalized to the measured activities in the uninhibited control reactions (black-filled bars; taken as 100% and corresponding to the dashed line in each panel). Error bars represent standard deviations for triplicate measurements.

As expected, each specific inhibitor exhibited between 84 and 100% inhibition of protease activity for its given substrate (Fig. S2). Interestingly, papain and cathepsin, a cysteine peptidase of the papain protease family, were both completely inhibited (99–100%) by zVAD-FMK, a pan-caspase inhibitor. This observation was not entirely surprising since it has been suggested that zVAD-FMK can indeed inhibit papain and papain-like peptidases (Rozman-Pungercar *et al.*, 2003). However, unlike *H. volcanii* extracts, neither purified papain nor cathepsin demonstrated preferential catalytic cleavage of caspase tetrapeptide recognition sequences (Fig. 1 and Schotte *et al.*, 1999).

We observed nearly complete inhibition (95%) of caspase-8-like activity (IETDase) when *H. volcanii* extracts were treated with zVAD-FMK (Fig. 3), corroborating our previous findings (Bidle *et al.*, 2010). Likewise, zVAD-FMK severely inhibited (> 85%) *H. volcanii* caspase-4-like (LEVDase) and caspase-6-like (VEIDase) activities further supporting that this activity is produced by a caspase-like enzyme(s) despite possessing distinct sequence differences to classic caspase superfamily pro-

teins (Bidle *et al.*, 2010). Given zVAD-fmk is a broad pan-inhibitor of caspases that is capable of inhibiting different caspases, we further refined our analysis of caspase inhibition by challenging cell extracts with fluoromethylketone inhibitors with higher specificity to individual caspase activities (e.g. LEVD-fmk, VEID-fmk, and IETD-fmk). The response to these specific caspase inhibitors may shed more light on the specificity of the observed caspase activities. In each case, we observed a dose-dependent inhibition of LEVDase, VEIDase and IETDase activities by their respective inhibitors (Fig. 4), but the degree of inhibition with 50 μ M of individual inhibitors was considerably lower than that observed for 20 μ M zVAD-fmk (Fig. 3). Higher inhibition with zVAD-CHO over specific caspase inhibitors (e.g. IETD-CHO) has also been reported for 'phytaspases', plant proteases with caspase activity (Chichkova *et al.*, 2010). For comparison, application of zVAD-fmk and IETD-fmk inhibited the activity of purified, recombinant human caspase-8 by 99.9% and 99.7% respectively (Fig. 4). Our findings suggest that the observed caspase activities in *H. volcanii* are not as refined as in higher eukaryotes and may represent a

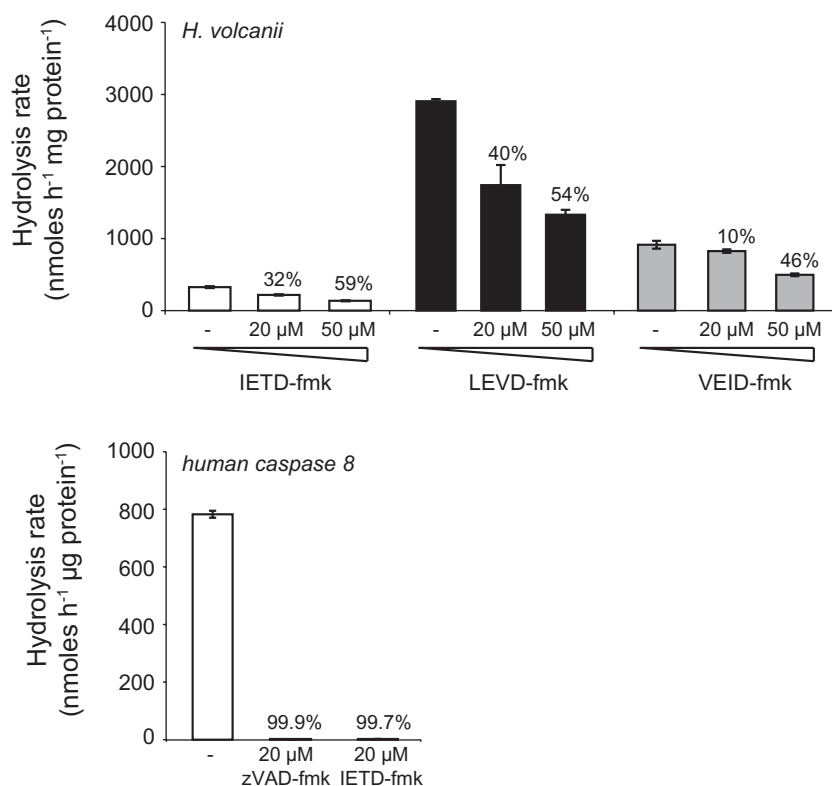


Fig. 4. Efficacy of specific fluoromethylketone inhibitors at abolishing caspase-specific activity. Caspase-specific activity in *H. volcanii* cell extracts was measured for IETD-AMC (open bars), LEVD-AMC (black bars), and VEID-AMC (grey bars) in the presence of IETD-fmk, LEVD-fmk and VEID-fmk respectively, at three different concentrations (0, 20, and 50 μM; Enzo Life Sciences). The percent inhibition at each respective concentration (relative to the untreated control) is indicated. For comparison, recombinant human caspase-8 was completely inhibited (> 99%) at 20 μM with both zVAD-fmk and IETD-fmk. Error bars represent standard deviations for triplicate measurements.

broader, more ancestral type of activity (Fig. 4). These results are also consistent with the presence of either one or several proteins with overlapping activities and/or functions.

Interestingly, EDTA partially inhibited the observed caspase activities derived from all three substrates tested (by 40–50%), indicating a possible requirement for magnesium ions (Rodriguez-Valera, 1995). *Haloflex volcanii* has a very high tolerance for MgCl₂ (Mullakhanbhai and Larsen, 1975), likely reflective of the abundant MgCl₂ concentrations found in the Dead Sea, which constitute 50.8% of the anhydrous chlorides on a weight percentage basis, compared with 30.4% for NaCl (Steinhorn, 1983). This is remarkably different from the composition of ocean seawater which is ~ 97% NaCl. Addition of 100 μM MgCl₂ to EDTA-treated cell extracts partially reconstituted caspase activity levels for all three substrates tested, but it was not able to restore all activity. MgCl₂ addition consistently elevated activity more than the addition of 100 μM CaCl₂ (Fig. 5) albeit slightly, thereby providing some support for a Mg²⁺ requirement.

Potential cellular roles

Our findings firmly root extremely high levels of specific caspase activity, particularly resembling caspase-4, as the dominant proteolytic activity in this extreme halophilic archaeon. These findings, along with our previous work

that linked very high basal caspase activity to the cellular salt stress response (Bidle *et al.*, 2010) raise intriguing questions as to the identity, function, and evolution of these caspase-associated proteins. We suggest that this caspase-activity may have emerged in *Archaea*, for essential housekeeping functions and possibly served as a foundation for stress and PCD pathways in higher organisms. In higher eukaryotes, caspase-4 activity has been implicated in endoplasmic reticulum (ER) stress (Kim *et al.*, 2006; Binet *et al.*, 2010), which can activate the 'unfolded protein response' (UPR) under conditions that alter protein folding or calcium homeostasis (Patil and Walter, 2001). UPR leads to the increased expression of chaperones and folding enzymes, which prevent the aggregation of misfolded proteins and facilitate proper protein folding. UPR can also induce components of the protein degradative machinery to remove misfolded proteins, which are tagged by ubiquitin and degraded in the proteasome. This physiological response affords the cell time to survive a stressful insult, unless enhanced or prolonged stress initiates PCD. Interestingly, eukaryotic proteasomes have been shown to contain caspase-like sites and associated activity (Kisselev *et al.*, 2003; Murata *et al.*, 2009). Archaeal proteasomes, which demonstrate a high level of structural similarity to those found in eukaryotes (Maupin-Furrow *et al.*, 2005) have been well studied in *H. volcanii* and play an integral role in the organisms' cellular stress response (Zhou *et al.*, 2008). Could the

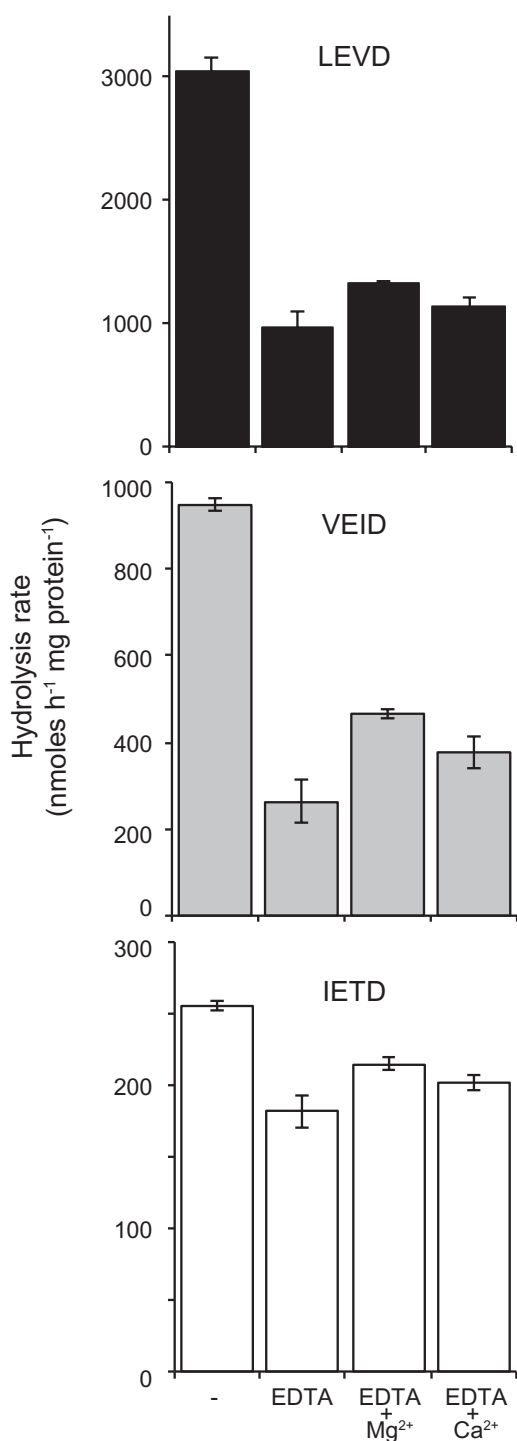


Fig. 5. Caspase catalytic activity in *H. volcanii* is stimulated by magnesium. Addition of 100 μM MgCl_2 to EDTA-treated cell extracts partially reconstituted activity but did not fully recover activity to levels of uninhibited cell extracts (control). Addition of 100 μM CaCl_2 to EDTA-treated cell extracts did not stimulate caspase activity in EDTA-treated extracts to the same degree. MgCl_2 and CaCl_2 were added to EDTA-treated cell extracts 1 h prior to the addition of fluorogenic substrates (LEVD-, VEID-, and IETD-AMC; indicated). Error bars represent standard deviations for triplicate measurements.

high-level caspase activity be linked to proteasomes and an ancestral UPR response? A UPR is present in all eukaryotes studied to date, but little is known if a similar response operates in *Archaea*. Regulation of proper protein folding in the face of very high extra- and intracellular salt environments encountered during growth is a critical concern for *H. volcanii* (and other extreme halophiles). Our results highlight the need to identify the protein(s) that confer caspase activity and function. Given the high specificity of *H. volcanii* to caspase catalytic activity our findings strongly suggest that recently developed in situ inhibitor trapping techniques (Tu *et al.*, 2006; Mohr and Zwacka, 2007) are a promising avenue to identify these enigmatic proteins and begin to elucidate their physiological roles and molecular evolution.

Acknowledgements

We thank Dr Chris Brown, Liti Haramaty, and Frank Natale for technical assistance and helpful discussions. This research was supported by NSF grants (OCE-0927829 and OCE-1061883) to K. D. B.

References

- Ameisen, J.C. (2002) On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. *Cell Death Differ* **9**: 367–393.
- Barrett, A.J., and Rawlings, N.D. (2007) ‘Species’ of peptidases. *Biol Chem* **388**: 1151–1157.
- Berman-Frank, I., Bidle, K., Haramaty, L., and Falkowski, P. (2004) The demise of the marine cyanobacterium, *Trichodesmium* spp., via an autocatalyzed cell death pathway. *Limnol Oceanogr* **49**: 997–1005.
- Bidle, K.A., Kirkland, P.A., Nannen, J., and Maupin-Furlow, J.A. (2008) Proteomic analysis of *Haloferax volcanii* reveals salinity-mediated regulation of the stress response protein PspA. *Microbiology* **154**: 1436–1443.
- Bidle, K.A., Haramaty, L., Baggett, N., Nannen, J., and Bidle, K.D. (2010) Tantalizing evidence for archaeal caspase-like protein expression and activity and its role in cellular stress response. *Environ Microbiol* **12**: 1161–1172.
- Bidle, K.D., and Bender, S.J. (2008) Iron starvation and culture age activate metacaspases and programmed cell death in the marine diatom, *Thalassiosira pseudonana*. *Eukaryot Cell* **7**: 223–236.
- Bidle, K.D., and Falkowski, P.G. (2004) Cell death in planktonic photosynthetic microorganisms. *Nat Rev Microbiol* **2**: 643–655.
- Bidle, K.D., and Kwityn, C.J. (2012) Assessing the role of metacaspase expression and caspase activity on viral susceptibility of the coccolithophore, *Emiliania huxleyi*. *J Phycol* **48**: 1079–1089.
- Bidle, K.D., Haramaty, L., Barcelos-Ramos, J., and Falkowski, P.G. (2007) Viral activation and recruitment of metacaspases in the unicellular coccolithophorid, *Emiliania huxleyi*. *Proc Natl Acad Sci USA* **104**: 6049–6054.

- Binet, F., Chiasson, S., and Girard, D. (2010) Evidence that endoplasmic reticulum (ER) stress and caspase-4 activation occur in human neutrophils. *Biochem Biophys Res Commun* **391**: 18–23.
- Chichkova, N.V., Shaw, J., Galiullina, R.A., Drury, G.E., Tuzhikov, A.I., Kim, S.H., *et al.* (2010) Phytaspase, a relocatable cell death promoting plant protease with caspase specificity. *EMBO J* **29**: 1149–1161.
- Cikala, M., Wilm, B., Hobmayer, E., Böttger, A., and David, C.N. (1999) Identification of caspases and apoptosis in the simple metazoan Hydra. *Curr Biol* **9**: 959–962.
- Coffeen, W.C., and Wolpert, T.J. (2004) Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*. *Plant Cell* **16**: 857–873.
- Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem J* **326**: 1–16.
- Englard, S., and Seifer, S. (1990) Precipitation techniques. In *Methods in Enzymology: Guide to Protein Purification*. Deutscher, M.P. (ed.). San Diego, CA, USA: Academic Press, pp. 285–300.
- Hartman, A.L., Norais, C., Badger, J.H., Delmas, S., Haldenby, S., Madupu, R., *et al.* (2010) The complete genome sequence of *Haloferax volcanii* DS2, a model archaeon. *PLoS ONE* **5**: e9605.
- Hedstrom, L. (2002) Serine protease mechanism and specificity. *Chem Rev* **102**: 4501–4524.
- Hoppe, H.-G., Arnosti, C., and Herndl, G.F. (2002) Ecological significance of bacterial enzymes in the marine environment. In *Enzymes in the Environment: Activity Ecology and Applications*. Burns, R.G., and Dick, R.P. (eds). New York, USA: Marcel Dekker, pp. 73–107.
- Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**: 239–257.
- Kim, S.J., Zhang, Z., Hitomi, E., Lee, Y.C., and Mukherjee, A.B. (2006) Endoplasmic reticulum stress-induced caspase-4 activation mediates apoptosis and neurodegeneration in INCL. *Hum Mol Genet* **15**: 1826–1834.
- Kisselev, A.F., Garcia-Calvo, M., Overkleeft, H.S., Peterson, E., Pennington, M.W., Ploegh, H.L., *et al.* (2003) The caspase-like sites of proteasomes, their substrate specificity, new inhibitors and substrates, and allosteric interactions with the trypsin-like sites. *J Biol Chem* **278**: 35869–35877.
- Koonin, E.V., and Aravind, L. (2002) Origin and evolution of eukaryotic apoptosis: the bacterial connection. *Cell Death Differ* **9**: 394–404.
- Lockshin, R.A., and Williams, C.M. (1965) Programmed cell death-I. Cytolytic enzymes in relation to the breakdown of the intersegmental muscles of silkworms. *J Insect Physiol* **11**: 831–844.
- Mace, P.D., Shirley, S., and Day, C.L. (2010) Assembling the building blocks: structure and function of inhibitor of apoptosis proteins. *Cell Death Differ* **17**: 46–53.
- Maupin-Furlow, J.A., Gil, M.A., Humbard, M.A., Kirkland, P.A., Li, W., Reuter, C.J., and Wright, A.J. (2005) Archaeal proteasomes and other regulatory proteases. *Curr Opin Microbiol* **8**: 720–728.
- Moharikar, S., D'Souza, J.S., Kulkarni, A.B., and Rao, B.J. (2006) Apoptotic-like cell death pathway is induced in unicellular Chlorophyte *Chlamydomonas reinhardtii* (Chlorophyceae) cells following UV irradiation: detection and functional analyses. *J Phycol* **42**: 423–433.
- Mohr, A., and Zwacka, R.M. (2007) In situ trapping of initiator caspases reveals intermediate surprises. *Cell Biol Int* **31**: 526–530.
- Mullakhanbhai, M.F., and Larsen, H. (1975) *Halobacterium volcanii* spec. nov., a Dead Sea halobacterium with a moderate salt requirement. *Arch Microbiol* **104**: 207–214.
- Murata, S., Yashiroda, H., and Tanaka, K. (2009) Molecular mechanisms of proteasome assembly. *Nat Rev Mol Cell Biol* **10**: 104–115.
- Otto, H.H., and Schirmeister, T. (1997) Cysteine proteases and their inhibitors. *Chem Rev* **97**: 133–171.
- Patil, C., and Walter, P. (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol* **13**: 349–355.
- Riedl, S.J., and Shi, Y. (2004) Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* **5**: 897–907.
- Robinson, J.L., Pyzyna, B., Atrasz, R.G., Henderson, C.A., Morrill, K.L., Burd, A.M., *et al.* (2005) Growth kinetics of extremely halophilic Archaea (family Halobacteriaceae) as revealed by Arrhenius plots. *J Bacteriol* **187**: 923–925.
- Rodriguez-Valera, F. (1995) Cultivation of halophilic Archaea. In *Halophiles*. DasSarma, S., and Fleischmann, E.M. (eds). Plainview, NY, USA: Cold Spring Harbor Laboratory Press, pp. 13–16.
- Rozman-Pungercar, J., Kopitar-Jerala, N., Bogyo, M., Turk, D., Vasiljeva, O., Stefe, I., *et al.* (2003) Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. *Cell Death Differ* **10**: 881–888.
- Salvesen, G.S., and Abrams, J.M. (2004) Caspase activation – stepping on the gas or releasing the brakes? Lessons from humans and flies. *Oncogene* **23**: 2774–2784.
- Sanmartín, M., Jaroszewski, L., Raikhel, N.V., and Rojo, E. (2005) Caspases. Regulating death since the origin of life. *Plant Physiol* **137**: 841–847.
- Schotte, P., Declercq, W., Van Huffel, S., Vandenaabeele, P., and Beyaert, R. (1999) Non-specific effects of methyl ketone peptide inhibitors of caspases. *FEBS Lett* **442**: 117–121.
- Segovia, M.Á., Haramaty, L., Berges, J.A., and Falkowski, P.G. (2003) Cell death in the unicellular chlorophyte *Dunaliella tertiolecta*: a hypothesis on the evolution of apoptosis in higher plants and metazoans. *Plant Physiol* **132**: 99–105.
- Steinhorn, I. (1983) In situ salt precipitation at the Dead Sea. *Limnol Oceanogr* **28**: 580–583.
- Stennicke, H.R., and Salvesen, G.S. (1998) Properties of the caspases. *Biochim Biophys Acta* **1387**: 17–31.
- Thornberry, N.A., and Lazebnik, Y. (1998) Caspases: enemies within. *Science* **281**: 1312–1316.
- Tu, S., McStay, G.P., Boucher, L.-M., Mak, T., Beere, H.M., and Green, D.R. (2006) In situ trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. *Nat Cell Biol* **8**: 72–77.
- Uren, A.G., O'Rourke, K., Pisabarro, M.T., Seshagiri, S.,

- Koonin, E.V., and Dixit, V.M. (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT Lymphoma. *Mol Cell* **6**: 961–967.
- Vardi, A., Berman-Frank, I., Rozenberg, T., Hadas, O., Kaplan, A., and Levine, A. (1999) Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO₂ limitation and oxidative stress. *Curr Biol* **9**: 1061–1064.
- Vartapetian, A.B., Tuzhikov, A.I., Chichkova, N.V., Taliany, M., and Wolpert, T.J. (2011) A plant alternative to animal caspases: subtilisin-like proteases. *Cell Death Differ* **18**: 1289–1297.
- Vaux, D.L., and Korsmeyer, S.J. (1999) Cell death in development. *Cell* **96**: 245–254.
- Vercammen, D., Declercq, W., Vandenaabeele, P., and Breusegem, F.V. (2007) Are metacaspases caspases? *J Cell Biol* **179**: 375–380.
- Wendoloski, D., Ferrer, C., and Dyal-Smith, M.L. (2001) A new simvastatin (mevinolin)-resistance marker from *Haloarcula hispanica* and a new *Haloferax volcanii* strain cured of plasmid pHV2. *Microbiology* **147**: 959–964.
- Wilson, H.L., Aldrich, H.C., and Maupin-Furlow, J. (1999) Halophilic 20S proteasomes of the archeon *Haloferax volcanii*: purification, characterization, and gene sequence analysis. *J Bacteriol* **181**: 5814–5824.
- Yutin, N., Makarova, K.S., Mekhedov, S.L., Wolf, Y.I., and Koonin, E.V. (2008) The deep archaeal roots of eukaryotes. *Mol Biol Evol* **25**: 1619–1630.
- Zhou, G., Kowalczyk, D., Humbard, M.A., Rohatgi, S., and Maupin-Furlow, J.A. (2008) Proteasomal components required for cell growth and stress responses in the haloarchaeon *Haloferax volcanii*. *J Bacteriol* **190**: 8096–8105.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Panel of proteolytic activities in partially purified protein fractions. Hydrolysis rates were determined for cell extracts (CE) from exponentially growing *H. volcanii* cells, partially purified proteins obtained by sequential ammonium sulfate (AmSO₄) precipitation (20%, 40%, 60%, 80% and 100%), and non-AmSO₄-precipitable proteins (soluble). AmSO₄ was sequentially added to cell extracts at 4°C with constant rocking and incubated for 30 min at each step. Prior to each AmSO₄ addition, proteins were pelleted at 10 000 g. The supernatant, representing dissolved proteins, was transferred to a new tube and used for the next percent AmSO₄ addition. Protein pellets were resuspended in 100 mM Tris-Cl (pH 7.4)/150 nM NaCl and activity was measured using the aforementioned assay conditions and panel of fluorogenic substrates. Note difference in scale between caspase substrates (upper panel) and other protease substrates (lower panel). Hydrolysis of Leu-AMC was also measured but no activity was detected in any fraction. Error bars represent standard deviations for triplicate measurements.

Fig. S2. Protease inhibitor efficacy on model protease substrates. To verify inhibitor effectiveness, purified proteases (recombinant human caspase-8, trypsin, papain, cathepsin B) were individually incubated with inhibitor treatments and compared with uninhibited control reactions. Each purified enzyme was pre-incubated with individual protease inhibitors at 37°C for 1 h at the aforementioned concentrations (see Fig. 3) prior to the addition of the preferred fluorogenic substrate for each respective enzyme: caspase-8 with IETD-, trypsin and cathepsin with ARR-, and papain with LLVY-AMC.