E1- and ubiquitin-like proteins provide a direct link between protein conjugation and sulfur transfer in archaea

Hugo V. Miranda[a], Nikita Nembhard[a], Dan Su[b], Nathaniel Hepowit[a], David J. Krause[a], Jonathan R. Pritz[a], Cortlin Phillips[a], Dieter Söll[b,c], and Julie A. Maupin-Furlow[a,1]

[a]Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611-0700; and [b]Department of Molecular Biophysics and Biochemistry, and [c]Department of Chemistry, Yale University, New Haven, CT 06511

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Based on our recent work with *Haloferax volcanii*, ubiquitin-like (Ubl) proteins (SAMP1 and SAMP2) are known to be covalently attached to proteins in archaea. Here, we investigated the enzymes required for the formation of these Ubl-protein conjugates (SAMPylation) and whether this system is linked to sulfur transfer. Markerless in-frame deletions were generated in *H. volcanii* target genes. The mutants were examined for: (*i*) the formation of Ubl protein conjugates, (*ii*) growth under various conditions, including those requiring the synthesis of the sulfur-containing molybdenum cofactor (MoCo), and (*iii*) the thiolation of tRNA. With this approach we found that UbaA of the E1/MoeB/ThiF superfamily was required for the formation of both SAMP1- and SAMP2-protein conjugates. In addition, UbaA, SAMP1, and MoaE (a homolog of the large subunit of molybdopterin synthase) were essential for MoCo-dependent dimethyl sulfoxide reductase activity, suggesting that these proteins function in MoCo-biosynthesis. UbaA and SAMP2 were also crucial for optimal growth at high temperature and the thiolation of tRNA. Based on these results, we propose a working model for archaea in which the E1-like UbaA can activate multiple Ubl SAMPs for protein conjugation as well as for sulfur transfer. In sulfur transfer, SAMP1 and SAMP2 appear specific for MoCo biosynthesis and the thiolation of tRNA, respectively. Overall, this study provides a fundamental insight into the diverse cellular functions of the Ubl system.

posttranslational modification | tRNA modification | proteasomes

The posttranslational conjugation of one or more molecules of ubiquitin (Ub) and ubiquitin-like (Ubl) proteins to selected proteins plays an integral role in a wide variety of functions in eukaryotic cells. Many of these processes are central to cell physiology, including the regulation of gene expression, heterochromatin formation, genome stability, protein trafficking, cell division, morphogenesis, DNA repair, autophagy, and proteasome-mediated proteolysis (1, 2).

Elaborate ATP-dependent conjugation systems covalently and reversibly attach Ub (and Ubl) proteins to their protein targets (3). In this cascade, the C terminus of Ub is adenylated by an Ub-activating E1 enzyme, which activates Ub for nucleophilic attack by the active site cysteine of E1 to generate an E1-Ub thioester. An E2 Ub-conjugating enzyme accepts this activated form of Ub on its active site cysteine to form the second thioester linkage in this pathway. From E2, Ub is transferred to the ε-amino group of a lysine residue either within the target protein or the growing poly-Ub chain, thus forming an isopeptide bond. Transfer is often assisted by an E3 Ub-ligase, either forming a transient E3-Ub thioester intermediate or facilitating the transfer of Ub from E2 directly to the substrate protein.

Ub and Ubl-proteins have a β-grasp fold that is common to a superfamily of proteins found in all three domains of life (4, 5). Proteins with this fold are diverse and mediate a variety of functions beyond the ubiquitination of proteins. For example, (*i*) 2[Fe-S] ferredoxins facilitate electron transfer, (*ii*) ThrRS, GTPase, and SpoT domains of tRNA synthetases are required for RNA-protein interactions, (*iii*) ThiS/MoaD proteins serve as sulfur carriers in thiamine and tungsten/molybdenum cofactor (W/MoCo) biosynthesis, and (*iv*) Atg8 and LC3 are conjugated to phospholipids (5–8).

β-Grasp fold-proteins that function as sulfur carriers and protein modifiers share a common chemistry. Both types of proteins are adenylated at their C terminus by an ATP-dependent E1/MoeB/ThiF-type enzyme (8, 9). This adenylation activates the β-grasp fold-protein for either the acceptance of sulfur as a C-terminal thiocarboxylate or the formation of an E1-Ubl thioester intermediate for subsequent protein modification.

Urm1, one of the most ancestral of eukaryotic Ubl-proteins, along with its E1-activating enzyme Uba4p (MOCS3) provide the only example to date of a system that functions in both protein conjugation (urmylation) (10, 11) and sulfur transfer (2-thiolation of tRNAs) (12–18). Uba4p adenylates and transfers sulfur to the C terminus of Urm1, resulting in the formation of a C-terminal thiocarboxylated form of Urm1 (14) that is required for both tRNA thiolation and urmylation (19). Although lysine residues of protein substrates and an apparent thioester intermediate are required for urmylation (19), the detailed chemistry of how Uba4p forms these covalent adducts and differentiates between protein conjugation and sulfur transfer remain to be determined.

Recently, we identified two Ubl proteins (SAMP1 and SAMP2) that are differentially conjugated to proteins in the haloarchaeon *Haloferax volcanii* (20). The SAMP2 C-terminal carboxylate was demonstrated to form an isopeptide bond with the ε-amino group of lysine residues on target proteins. Although related to ubiquitination, this type of posttranslational modification (SAMPylation) appears to be simplified in comparison. Like *H. volcanii*, all archaea encode Ubl proteins. However, only a single member of the E1/MoeB/ThiF superfamily is present and E2- and E3-like proteins are not predicted in the majority of archaeal genomes (8).

Here we demonstrate that the E1-like UbaA and Ubl SAMPs of *H. volcanii* are required for protein conjugation and sulfur transfer, including the thiolation of tRNA and most likely MoCo biosynthesis. Thus, archaeal Ubl-systems are highly versatile in their function and provide an example of a Ubl-system that forms not only classic isopeptide bonds with diverse protein targets, but also mediates sulfur transfer.

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[1]To whom correspondence should be addressed. E-mail: jmaupin@ufl.edu.

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Results and Discussion

Identification of Putative Genes Required for SAMPylation. Similar to most archaea, *H. volcanii* encodes a single protein of the E1/MoeB/ThiF superfamily (HVO_0558), which we have termed Ubl-activating enzyme of archaea or UbaA for its potential role in activating SAMPs. Our previous work identified UbaA conjugated to SAMP1 and SAMP2 by MS-based proteomics (20). To further understand the function of UbaA, its protein sequence was compared with E1/MoeB/ThiF-type homologs by multiple amino acid sequence alignment (Fig. 1 and *SI Appendix*, Fig. S1). Based on this alignment, UbaA had all of the conserved residues needed to catalyze the ATP-dependent adenylation of the SAMPs, including a glycine-rich region related to the NTPase P-loop motif. In addition, UbaA C188 was analogous to the active site cysteine required for the formation of the E1-Ub thioester intermediate of ubiquitination in eukaryotes (21) and the acyldisulfide-linked ThiF-ThiS intermediate of thiamine biosynthesis in bacteria (22). UbaA also had four conserved cysteine residues predicted to coordinate Zn^{2+} for structural integrity similar to E1-like enzymes. However, UbaA did not have the extended C-terminal rhodanese domain (RHD) that is common to Uba4p and MOCS3. Instead, like other archaea, the *H. volcanii* RHDs are separately encoded by multiple genes including *ubaB* (HVO_0559) that is divergently transcribed from *ubaA* (*SI Appendix*, Fig. S2).

In addition to UbaA, archaea encode homologs of MoaE (MOCS2B) (*SI Appendix*, Fig. S3), known in bacteria (and eukaryotes) to associate with the Ubl MoaD (MOCS2A) (23). This association occurs after the C terminus of MoaD is adenylated by the E1-like MoeB and thiocarboxylated by the IscS cysteine desulfurase. Molybdopterin (MPT) synthase is a heterotetramer of MoaE and thiocarboxylated MoaD (MoaD-COSH) that catalyzes the transfer of sulfur from the bound MoaD-COSH to cyclic pyranopterin monophosphate (cPMP or precursor Z). This catalyzation forms the dithiolene moiety of MPT before the insertion of molybdenum. In methanogenic and halophilic archaea, MoaE is often fused to an N-terminal P-loop NTPase MobB domain (*e.g.*, HVO_1864 of *H. volcanii*, termed MoaE) (*SI Appendix*, Fig. S3). MobB functions downstream of MoaE in bacteria to form molybdopterin guanine dinucleotide common to enzymes of the DMSO reductase family (24, 25). The MoaE homolog of *H. volcanii* and other archaea is likely to be linked to Ubl protein function, based on its amino acid sequence relationship to the large subunit of MPT synthase and its association with SAMP1 as detected by MS (20).

Gene Knockout to Investigate the Relationship of Protein Conjugation and Sulfur Transfer in Archaea. To further investigate the roles of the archaeal Ubl SAMPs and to identify enzymes catalyzing their activation and covalent modification, a series of knockout strains was generated in the *H. volcanii* genome using the markerless *pyrE2*-based deletion strategy (26, 27) (strains listed in *SI Appendix*, Table S1). Targets for deletion were the genes encoding SAMP1 and SAMP2, as well as genes implicated in SAMPylation based on comparative genomics (discussed above) and MS-based proteomics (20). This latter set included genes encoding UbaA (*HVO_0558*), MoaE (*HVO_1864*), and UbaB (*HVO_0559*). In addition, a gene (*HVO_2177*), encoding a third Ubl protein that has a β-grasp fold and C-terminal diglycine motif similar to SAMP1 and SAMP2, was deleted in single and triple knockout with the SAMP1 and SAMP2 genes. Mutant strains were confirmed by Southern blot, PCR and RT-qPCR (for details see *SI Appendix*, Tables S1–S3 and Figs. S4 and S5).

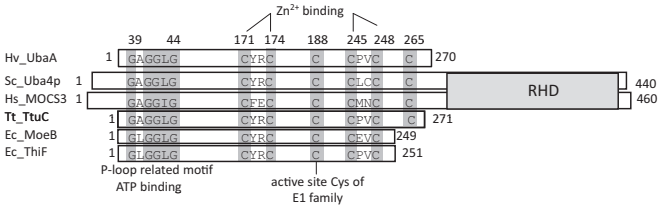

Fig. 1. Schematic representation of the *H. volcanii* (Hv) UbaA with members of the MoeB/E1 superfamily. The glycine rich region related to the NTPase P-loop motif, conserved cysteine residues, and C-terminal RHD are indicated with conserved amino acid residues shaded and numbered according to UbaA (HVO_0558). Uba4p of yeast (*Saccharomyces cerevisiae*, Sc), MOCS3 of human (*Homo sapiens*, Hs), TtuC of *Thermus thermophilus* (Tt), and ThiF and MoeB of *Escherichia coli* (Ec) are presented for comparison.

UbaA Is Required for SAMPylation. To examine whether *ubaA*, *moaE*, or *ubaB* are required for SAMPylation, parent and mutant strains were transformed with plasmids expressing Flag-SAMP1 and Flag-SAMP2 and grown under conditions known to enhance protein-conjugate levels (aerobically with *N*-limitation) (20). Cellular protein was separated by reducing SDS/PAGE and analyzed by α-Flag immunoblot. With this approach, we found that the *moaE* and *ubaB* genes were not required for the formation of SAMP protein-conjugates (Fig. 2 *A* and *B*). In contrast, the *ubaA* mutant was deficient in SAMPylation with unconjugated Flag-SAMP1 and Flag-SAMP2 proteins detected at 15 to 20 kDa (Fig. 2*A*), in a pattern similar to previously reported for the SAMPs synthesized with C-terminal diglycine motif deletions (ΔGG) (20). The reason for the doublet of SAMP2-specific bands (at 16 and 17 kDa) in the *ubaA* mutant is unclear, yet comparable to cells expressing SAMP2ΔGG (20). The *ubaA* knockout was complemented by providing a wild-type copy, but not a C188S variant of *ubaA in trans* (Fig. 2 *C* and *D*), with production of these UbaA proteins confirmed by immunoblot (*SI Appendix*, Fig. S7). Thus, UbaA Cys188 appears to function as an active site residue in SAMPylation similar to E1-type enzymes.

UbaA, MoaE, and SAMP1 Appear Crucial for MoCo Biosynthesis. Growth of mutant strains, including single knockouts of *ubaA*, *moaE*, and *ubaB*, as well as single and triple knockouts of the Ubl genes (*samp1*, *samp2*, and *hvo_2177*), was investigated in rich media at optimal growth temperature (42 °C) in the presence of either oxygen or DMSO as the terminal electron acceptor. In the presence of oxygen, growth was relatively similar to wild-type for all mutant strains examined (*SI Appendix*, Fig. S8). Likewise, growth rates were similar to wild-type for the *samp2*, *hvo_2177*, and *ubaB* single knockouts under anaerobic conditions with DMSO (Fig. 3*A* and *SI Appendix*, Fig. S9). In contrast, mutants with deletions in *ubaA*, *moaE*, or *samp1* did not grow anaerobically with DMSO but could be complemented by providing a wild-type copy of the corresponding gene *in trans* (Fig. 3). The gene encoding UbaA C188S did not complement the *ubaA* knockout (Fig. 3*B*), revealing that Cys188 is likely to be important for the catalytic function of UbaA under these conditions. Furthermore, plasmids expressing Flag-SAMP2 and Flag-HVO_2177 did not complement the *samp1* mutation (Fig. 3*A*), suggesting the role of SAMP1 is distinct from these two other Ubl β-grasp fold-proteins during anaerobic growth on DMSO.

One possibility for the lack of growth of the *ubaA*, *moaE*, and *samp1* mutants on DMSO is that these genes are required for sulfur transfer to cPMP to form the dithiolene intermediate in MoCo biosynthesis. This possibility would be in analogy to the *Escherichia coli moeB*, *moaE*, and *moaD* genes required to generate MoCo for the catalytic subunit of DMSO reductase (DmsA). To further investigate this, the *ubaA*, *moaE*, and *samp1* mutant and wild-type strains were grown aerobically to log-phase and then incubated under anaerobic conditions with DMSO. Cell lysate was assayed for DMSO reductase activity, and total RNA was analyzed for *dmsA*-specific transcript levels by RT-PCR. Although DMSO reductase activity was readily detected in the parent and complemented strains, it was not detected in the *ubaA*, *moaE*, and *samp1* mutants (Table 1). The *dmsA*-specific transcript, however, was present in all strains examined (*SI Appendix*, Fig. S10), sug-

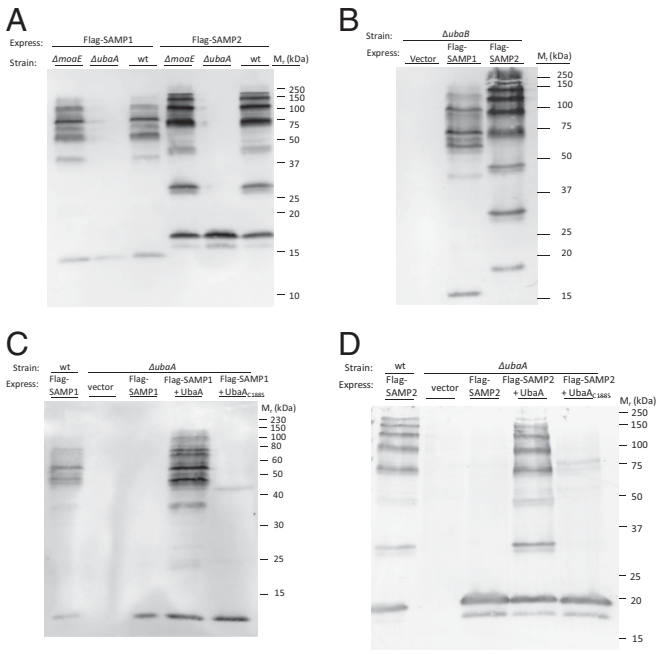

Fig. 2. UbaA is required for SAMPylation. Deletion of *ubaA* reduces the level of SAMP1- and SAMP2- protein conjugates in the cell (*A*), *moaE* and *ubaB* are not required for formation of these conjugates (*A* and *B*), and the *ubaA* mutation is complemented by providing a wild-type copy of *ubaA in trans* (*C* and *D*). *H. volcanii* strains (indicated above, where wild-type represents parent H26) were grown aerobically to stationary phase in *N*-limiting medium. Cell lysate was separated by reducing SDS/PAGE. Flag-SAMP proteins and conjugates were detected by α-Flag immunoblot. Equivalent protein loading was confirmed by staining parallel gels for total protein with Coomassie blue (*SI Appendix*, Fig. S6). Molecular mass standards are indicated on the right.

gesting UbaA, SAMP1, and MoaE are required for MoCo biosynthesis and, ultimately, maturation of the DmsA apoprotein into the MoCo-holoprotein. Although we do not rule out the possibility that SAMP1 modulates DMSO reductase through protein conjugation mechanisms, we propose a working model that UbaA can activate SAMP1 to function as a sulfur carrier with MoaE for MPT synthesis.

To bolster our proposal, we analyzed the levels of SAMP1 and SAMP2 protein-conjugates formed after cells were grown on rich medium in the presence of oxygen or DMSO (*SI Appendix*, Fig. S11). Similar to our previous work (20), the levels of both types of SAMP protein-conjugates appeared low when cells were grown on rich medium in the presence of oxygen, with the majority of Flag-SAMP proteins at 15 to 20 kDa in the unconjugated form. Anaerobic growth with DMSO as the terminal electron acceptor did not enhance the levels of SAMP1 protein-conjugates, suggesting SAMP1ylation is not a major mechanism of posttranslational protein modification under these conditions. Instead, the levels of SAMP2 protein-conjugates were relatively high under anaerobic conditions with DMSO and reached levels comparable to that previously observed under *N*-limiting conditions in the presence of oxygen (20). For cells grown in the presence of oxygen, addition of glucose to the complex medium had a stimulatory effect on SAMP2ylation. However, these levels were modest compared with the robust levels of SAMP2ylation observed during anaerobic growth with DMSO, and may be because of the reduced levels of oxygen likely to occur from the enhanced growth observed upon glucose supplementation.

UbaA and SAMP2 Are Needed for Optimal Growth at High Temperature. To investigate whether UbaA, SAMP, and other SAMP-associated proteins are needed for optimal growth at high temperature, the aerobic growth of *H. volcanii* parent and single knockout strains was monitored on rich medium at temperatures equal to and above the 42 to 45 °C optimum (28). Although all strains grew similar to wild-type at 42 °C, *ubaA* and *samp2* mutants were retarded in growth at 50 °C (*SI Appendix*, Fig. S12). Although many factors may be responsible for this phenotype, such as deficiencies in proteasome function (29), it is interesting to note that the *samp1* and other associated mutant strains that we examined were not hypersensitive to growth at high temperature. Based on our earlier work, it is the SAMP1 (and not SAMP2) protein-conjugates that accumulate in proteasomal mutant strains, whereas SAMP2 modifies homologs of the Urm1 pathway including Tum1 (Yor251cp) and Ncs6p, both of which are important in the thiolation of tRNA (20). Interestingly, mutant strains deficient in the ability to thiolate tRNA are often temperature-sensitive (e.g., ref. 30). Thus, the temperature-sensitive phenotype of *ubaA* and *samp2* mutants may be because of reduced thiolation of tRNAs.

UbaA and SAMP2 Are Required for the Thiolation of tRNA. To investigate the role of the SAMPs and UbaA in the thiolation of tRNA, total RNA isolated from mutant, parent, and complemented strains was: (*i*) separated by [(*N*-acryloylamino)phenyl] mercuric chloride (APM) gel electrophoresis and (*ii*) hybridized to a probe specific for lysine tRNAs with anticodon UUU ($tRNA^{Lys}_{UUU}$) (Fig. 4). This probe was selected based on the widespread distribution of 2-thiouridine derivatives in the first position or "wobble base" in the anticodon of lysine tRNAs (in addition to glutamine and glutamate tRNAs) (31). The majority of the $tRNA^{Lys}_{UUU}$ pool of parent H26 and *samp1*, *hvo_2177*, and *ubaB* mutant strains was found to be thiolated (Fig. 4), based on its retarded migration in APM gels (32). In contrast, the $tRNA^{Lys}_{UUU}$ of *ubaA* and *samp2* mutant strains appeared nonthiolated with this defect restored in part by expression of the corresponding genes *in trans*. The *ubaA* mutant was not complemented by the gene encoding UbaA C188S, suggesting this cysteine residue is important for UbaA function in the tRNA thiolation pathway.

Working Model for Ubl Proteins in Archaea. Based on our current and previous findings, we propose a working model for archaea in which the E1-like UbaA and Ubl SAMP proteins function in both protein conjugation and sulfur transfer (Fig. 5). In this model, UbaA catalyzes the adenylation of the C-terminal glycine of the SAMPs for their activation in protein conjugation. This adenylation would also activate SAMP1 and SAMP2 for their acceptance of sulfur as a C-terminal thiocarboxylate to serve as a sulfur carrier in MoCo biosynthesis and tRNA thiolation, respectively.

During protein conjugation, a thioester intermediate is suggested to be formed between the active site Cys188 of UbaA and the C-terminal carboxyl group of the SAMPs. This prediction is based on: (*i*) the requirement of UbaA Cys188 for protein-conjugate formation, (*ii*) the conservation of UbaA Cys188 with the active site cysteine of E1-type enzymes known to form an E1-Ub thioester, and (*iii*) the detection of isopeptide (and not persulfide) bonds between the C-terminal carboxyl group of SAMP2 and the ε-amino group of lysine residues of target proteins. However, further studies are needed to demonstrate this intermediate.

It is not clear whether UbaA forms a covalent intermediate with the SAMPs after their adenylation in the sulfur transfer pathways and what provides the activated source of sulfur for this putative thiocarboxylation reaction. The requirement of UbaA Cys188 for anaerobic growth on DMSO and tRNA thiolation suggests this residue is important in sulfur-transfer pathways and may form either a thioester or acyldisulfide intermediate with the SAMPs. This latter intermediate would be analogous to the E1-like ThiF of *E. coli* that forms an acyldisulfide conjugate with the Ubl ThiS in thiamine biosynthesis (22), but contrasts with the E1-like MoeB of *E. coli* that appears to form only noncovalent bonds with the Ubl MoaD during MoCo biosynthesis (33). Ultimately, C-terminal thiocarboxylated forms of the SAMPs are proposed to be generated after their activation by UbaA. In many archaea, the source of

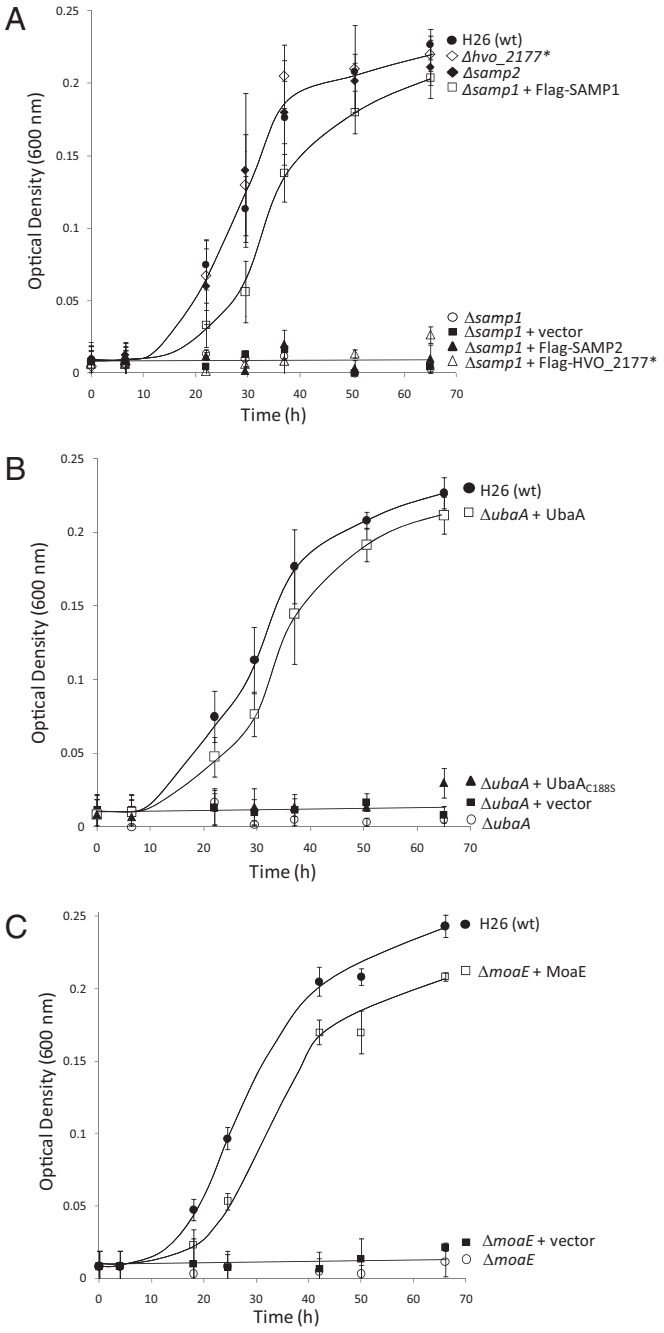

Fig. 3. SAMP1, UbaA, and MoaE are required for anaerobic growth on DMSO (*A*, *B*, and *C*, respectively). *H. volcanii* strains (indicated on the right) were grown on rich medium (YPC) with glucose supplementation and DMSO as the external electron acceptor, as described in *Materials and Methods*. Growth was monitored over time by an increase in OD_{600}. Similar results were observed during growth on ATCC 974 or YPC media with glycerol and DMSO supplementation, and growth was not observed for any strains under anaerobic conditions in the absence of DMSO.

Table 1. UbaA, SAMP1, and MoaE are required for DMSO reductase activity of *H. volcanii* cells

Strain-plasmid	Description	DMSO reductase Specific activity (U·mg protein^{-1})
H26	Parent	0.240 ± 0.029
HM1052	Δ*ubaA*	UD
HM1052-pJAM202c	Δ*ubaA* + vector	UD
HM1052-pJAM957	Δ*ubaA* + UbaA	0.358 ± 0.032
HM1052-pJAM1116	Δ*ubaA* + UbaA C188S	UD
HM1041	Δ*samp1*	UD
HM1041-pJAM202c	Δ*samp1* + vector	UD
HM1041- pJAM947	Δ*samp1* + SAMP1	0.255 ± 0.024
HM1053	Δ*moaE*	UD
HM1053-pJAM202c	Δ*moaE* + vector	UD
HM1053-pJAM1119	Δ*moaE* + MoaE	0.227 ± 0.026

UD, undetectable. Cell growth and assay of DMSO reductase activity are described in *Materials and Methods*. UbaA, UbaA C188S, and MoaE are synthesized as C-terminal StrepII tag fusions. SAMP1 is synthesized as a fusion with an N-terminal Flag-tag.

sulfur for this thiocarboxylation is likely to be derived from cysteine through a persulfide intermediate of a NifS/IscS-type cysteine desulfurase. However, not all archaea generate free pools of cysteine (e.g., *Methanococcus maripaludis*) (34, 35), and instead may use other forms of sulfur for this thiocarboxylation reaction or not require SAMPs as sulfur carrier proteins. Whether RHD proteins provide a sulfur relay between NifS/IscS-type proteins during the thiocarboxylation of the SAMPs similar to the RHD Tum1 (YOR251c) (12) of tRNA thiolation in yeast is also unclear. In *H. volcanii*, the RHD protein UbaB is not required for protein conjugation, MoCo biosynthesis, or the generation of thiolated $tRNA^{Lys}$. However, other RHD proteins may function in this sulfur relay or compensate for the loss of UbaB function in the *ubaB* mutant.

The interaction partners that facilitate sulfur transfer from the thiocarboxylated SAMP proteins to the biosynthetic intermediates are likely to be specific for each of the different sulfur transfer pathways. In MoCo biosynthesis, MoaE is proposed to form a complex with the thiocarboxylated form of SAMP1 and facilitate sulfur transfer to precursor Z. This proposal is based on: (*i*) the detection of MoaE with SAMP1 (and not SAMP2) as a complex by MS-based proteomics (20), (*ii*) the requirement of MoaE for DMSO reductase activity and not protein-conjugation, and (*iii*) the similarity of MoaE and SAMP1 to the subunits of the MPT synthase complex of *E. coli* and humans (36–40). In tRNA thiolation, the ATPase PP-loop superfamily member HVO_0580 is proposed to facilitate sulfur transfer from the SAMP2 thiocarboxyl to position 2 of the wobble uridine (U34) present in some tRNA species. HVO_0580 is homologous to tRNA modification enzymes, such as TtcA important in cytidine 2-thiolation of tRNA in bacteria (39), and Ncs6p/Ncs2p, implicated in uridine 2-thiolation of tRNA in

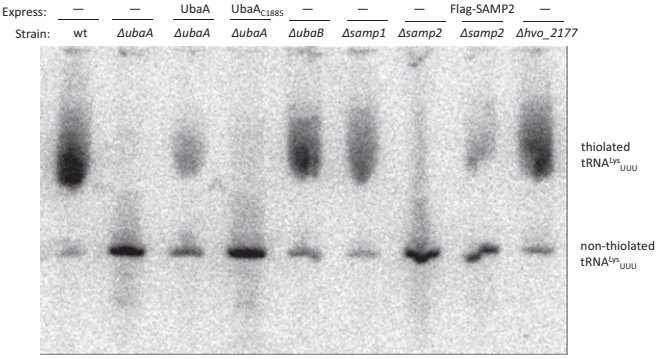

Fig. 4. UbaA and SAMP2 are required for the thiolation of $tRNA^{Lys}_{UUU}$. Total RNA was isolated from *H. volcanii* strains (indicated above), separated by APM gel electrophoresis, and hybridized with a probe complementary to $tRNA^{Lys}_{UUU}$, as described in *Materials and Methods*. Thiolated $tRNA^{Lys}_{UUU}$ is based on its retardation in APM gels compared with the nonthiolated form, which migrates faster.

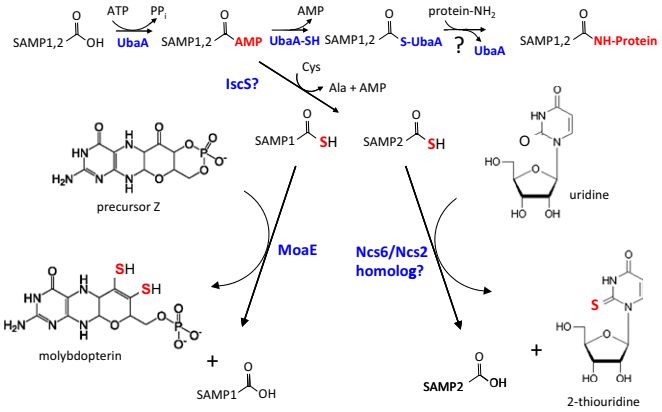

Fig. 5. Working model in which the E1-like UbaA and Ubl SAMP proteins function in both protein conjugation and sulfur transfer pathways in archaea (see text for details).

eukaryotes (12, 15, 18, 40). HVO_0580 also associates with SAMP2 (and not SAMP1) as determined by MS (20).

Although 26S proteasomes and the enzymes mediating ubiquitination are essential for eukaryotic cell division (reviewed in refs. 41 and 42), we observe differences in archaea. Like eukaryotes, 20S proteasomal core particles are required for archaeal cell division based on the inability of *H. volcanii* to grow when core particles (the single β-type or both α-type subunits of these complexes) are conditionally depleted from cells (29). However, in contrast to the core particles, neither the proteasome activating nucleotidase proteins (PANs, homologs of the regulatory particle triple-A or AAA ATPase particles of eukaryal 26S proteasomes) nor the SAMPylation system are essential for archaeal cell growth. This latter conclusion is based on the robust aerobic growth observed for *H. volcanii* cells even after knockout of both Rpt-like genes (*panA* and *panB*) (29), all three of the Ubl genes (*samp1*, *samp2*, and *hvo_2177*), or depletion of *ubaA* gene function. This discrepancy, between the core particles being essential and the AAA-ATPase PAN and SAMPylation pathway being dispensable for growth under aerobic conditions presents a few scenarios. First, it is likely that in archaea, not all proteins degraded by core particles are targeted by the Ubl-protein conjugation pathway (SAMPylation). It is also possible that not all proteasomal substrates require the Rpt-like PANs for entry into the core particle proteolytic chamber (e.g., homologs of Cdc48-like AAA ATPases common among archaea and associated with core particle function in eukaryotes may serve this alternative role). Finally, the core particles may mediate protein degradation in the absence of AAA ATPases and facilitate other cellular processes that are essential for cell division but independent of proteasome-mediated proteolysis.

Materials and Methods

Materials. Biochemicals and analytical-grade inorganic chemicals were purchased from Fisher Scientific, Bio-Rad, and Sigma-Aldrich. Desalted oligonucleotides were from Integrated DNA Technologies. DNA polymerases and modifying enzymes were from New England Biolabs. Hi-Lo DNA standards were from Minnesota Molecular, Inc.. APM was synthesized according to Igloi et al. (32).

Strains, Media, and Plasmids. The strains, primers and plasmids used in this study are summarized in *SI Appendix*, Tables S1 and S2. Site-directed mutagenesis was performed using a QuikChange Lightning kit according to the supplier (Stratagene). *E. coli* strains, DH5α used for routine recombinant experiments and GM2163 used for isolation of plasmid DNA for transformation of *H. volcanii* (43), were grown at 37 °C in Luria-Bertani medium. *H. volcanii* strains were grown at 42 °C (or 50 °C where indicated). Growth media included complex media (YPC and ATCC974) and glycerol minimal medium with alanine as the nitrogen source (GMM alanine), as previously described (20, 43). Ampicillin (0.1 mg·mL^{-1}), novobiocin (0.1 μg·mL^{-1}), and agar (1.5% wt/vol) were included as needed. For anaerobic growth, *H. volcanii* strains were twice grown aerobically on complex media to log-phase (2 mL in 13 × 100-mm tubes; 200 rpm) and inoculated at 1% (vol/vol) for anaerobic growth in 10-mL screw-cap tubes on complex media supplemented with 100 mM DMSO and 2% (wt/vol) glucose (or 20 mM glycerol where indicated). For analysis of DMSO reductase activity and *dsmA*-specific transcripts, *H. volcanii* strains were first grown aerobically in YPC medium (2 mL in 13 × 100-mm tubes, 200 rpm) and inoculated (1% vol/vol) into fresh YPC medium (3 × 50 mL in 250 mL baffled flasks, 200 rpm) to late-log (OD_{600} of 1.4–1.5 units). Cultures were pooled, supplemented with 100 mM DMSO and 2% (wt/vol) glucose and transferred to Wheaton bottles (150 mL). Bottles were sealed with butanol rubber stoppers and incubated overnight at 42 °C. Growth was monitored at OD_{600}.

Generation of Knockout Strains. Target genes were deleted from the *H. volcanii* chromosome using an established *pyrE2*-based pop-in/pop-out method (26, 27). Mutants were confirmed by PCR, DNA sequencing and Southern blot as previously described (29).

Immunoblot. Cells were harvested by centrifugation (14,000 × *g*, 10 min, 25 °C), resuspended in SDS loading buffer [100 mM Tris-Cl buffer at pH 6.8 with 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.6 mg·mL^{-1} bromophenol blue, and 2.5% (vol/vol) β-mercaptoethanol] and boiled for 20 to 30 min. Proteins were separated by SDS-PAGE (10 or 12%) and electroblotted onto PVDF membranes (Amersham). Equivalent protein loading was determined by OD_{600} of cell culture (0.065 units per lane) and confirmed by staining parallel gels with Coomassie blue. Epitope-tagged proteins were detected by immunoblot using alkaline phosphatase-linked anti-Flag M2 monoclonal antibody (Sigma) or rabbit anti-StrepII polyclonal antibody (GenScript) combined with goat anti-rabbit IgG (H+L)-alkaline phosphatase-linked antibody (SouthernBiotech). Alkaline phosphatase activity was detected colorimetrically using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and by chemiluminescence using CDP-Star (Applied Biosystems) with X-ray film (Hyperfilm; Amersham Biosciences).

DMSO Reductase Activity Assay. Cells (15 mL culture) were harvested by centrifugation (6,000 × *g*, 20 min at 4 °C), washed in 15 mL buffer A (50 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 2 M NaCl), resuspended in 1 mL buffer A, and lysed by sonication (4 × 20 s at 140 W). Cell lysate was clarified by centrifugation (14,000 × *g*, 20 min at 4 °C), and protein concentration was determined using the Bradford assay with BSA as a standard (BioRad). DMSO reductase activity was monitored at $A_{600\ nm}$ (15-s intervals for 3.5 min) with nitrogen as the headspace. Assays (4 mL) included cell lysate (1–1.5 mg protein) and 0.3 mM methyl viologen in buffer A. The mixture was titrated with fresh 20 mM sodium dithionite ($Na_2S_2O_4$) in 20 mM sodium bicarbonate ($NaHCO_3$) to 1 to 1.2 $A_{600\ nm}$ units before addition of 10 mM DMSO. One unit (U) of enzyme activity is defined as 1-μmol substrate consumed per minute at room temperature with an extinction coefficient $A_{600\ nm}$ of 13.6 (mM^{-1}·cm^{-1}) for methyl viologen. All assays were performed in biological triplicate with the means ± SDs calculated.

RNA Isolation. Total RNA was isolated for RT-(q)PCR (see *SI Appendix*, Figs. S5 and S10 for details) using the RNeasy Mini Kit (Qiagen) with a typical yield of 10 to 15 μg RNA per 1 mL culture. RNA samples were treated with RNase-free DNase (Qiagen) to remove genomic DNA contamination as confirmed by PCR. For tRNA thiolation assays, total RNA was extracted, as previously described (44), from log-phase cells grown in ATCC 974 medium (37.5 mL of 100 mL culture in a 500-mL flask; 42 °C at 200 rpm). RNA was further purified by extraction with equal volume of acidic-phenol (pH 5.0): chloroform: isoamyl alcohol (25:24:1) followed by chloroform: isoamyl alcohol (24:1). RNA was precipitated in 0.25 M sodium acetate (pH 5.0) with two volumes of 95% ethanol (−70 °C, 15 min) and washed with 70% ethanol. The air-dried RNA pellet was resuspended in 30 μL DEPC-treated water with a typical yield of 100 to 150 μg RNA. RNA quality was assessed by agarose gel electrophoresis, and RNA concentration was determined by $A_{260\ nm}$.

Assay for tRNA Thiolation. APM-gel retardation analysis of tRNA was performed as follows. Total RNA (10 μg per lane) was separated by electrophoresis using 12% urea-PAGE gels supplemented with 30 μg APM per milliliter. RNA was transferred to a Hybond N+ nylon membrane (GE Healthcare) and immobilized by UV-crosslinking before hybridization. The oligonucleotide probe (see *SI Appendix*, Table S1 for details) was 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Excess [γ-^{32}P]ATP was removed by passing the reaction through a MicroSpin G-25 column (GE Healthcare). The membrane was prehybridized in ULTRAhyb-Oligo buffer

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(Ambion) for 30 min at 42 °C before addition of the end-labeled oligonucleotide (~10^6 cpm per milliliter). After hybridization at 42 °C for 14 h, the membrane was washed twice with buffer consisting of 2× SSC and 0.5% SDS (for 30 min each time at 42 °C). The blot was exposed to an imaging plate (FujiFilm) and scanned on a Molecular Dynamics Storm 860 Phosphoimager (GE Healthcare).

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