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Proteome targets of ubiquitin-like samp1ylation are associated with sulfur metabolism and oxidative stress in *Haloferax volcanii*

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Abstract

Small archeal modifier proteins (SAMPs) are related to ubiquitin in tertiary structure and in their isopeptide linkage to substrate proteins. SAMPs also function in sulfur mobilization to form biomolecules such as molybdopterin and thiolated tRNA. While SAMP1 is essential for anaerobic growth and covalently attached to lysine residues of its molybdopterin synthase partner MoaE (K240 and K247), the full diversity of proteins modified by samp1ylation is not known. Here, we expand the knowledge of proteins isopeptide linked to SAMP1. LC-MS/MS analysis of -Gly-Gly signatures derived from SAMP1 S85R conjugates cleaved with trypsin was used to detect sites of sampylation (23 lysine residues) that mapped to 11 target proteins. Many of the identified target proteins were associated with sulfur metabolism and oxidative stress including MoaE, SAMP-activating E1 enzyme (UbaA), methionine sulfoxide reductase homologs (MsrA and MsrB), and the Fe-S assembly protein SufB. Several proteins were found to have multiple sites of sampylation, and the isopeptide linkage at SAMP3 lysines (K18, K55, and K62) revealed hetero-SAMP chain topologies. Follow-up affinity purification of selected protein targets (UbaA and MoaE) confirmed the LC-MS/MS results. 3D homology modeling suggested sampylation is autoregulatory in inhibiting the activity of its protein partners (UbaA and MoaE), while occurring on the surface of some protein targets, such as SufB and MsrA/B. Overall, we provide evidence that SAMP1 is a ubiquitin-like protein modifier that is relatively specific in tagging its protein partners as well as proteins associated with oxidative stress response.

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Keywords

Archaea; Microbiology; Oxidative stress; Sampylation; Sulfur mobilization; Ubiquitin

1 Introduction

Isopeptide linkage of small protein modifiers to target proteins regulates diverse cellular processes [1, 2]. One of the most widely studied protein modifiers is ubiquitin (Ub), which targets proteins for turnover by proteasomes [3, 4]. Nonproteolytic consequences of ubiquitin attachment have also been described, including vesicular protein trafficking, chromatin packing dynamics, and DNA repair [5, 6].

SAMPs are small archeal modifier proteins related to ubiquitin. Like ubiquitin, SAMPs have a tertiary β -grasp fold structure and C-terminal di-glycine (di-Gly) motif that is isopeptide linked to the μ -amino group of lysine residues of protein targets in a process termed sampylation [7, 8]. SAMPs are activated (adenylated) in an ATP-dependent manner by the E1-like UbaA/ELSA [9, 10] and liberated from SAMP conjugates by the action of the JAMM/MPN+ Zn^{2+} -metalloprotease HvJAMM1 [11].

Aside from functioning as protein modifiers, SAMPs are associated with sulfur mobilization, mirroring the sulfur-carrier proteins of bacterial and eukaryotic systems. SAMP2 is required to generate thiolated tRNA^{UUU}, while SAMP1 is suggested to mediate sulfur relay to form molybdopterin (MPT) from precursor Z in the molybdenum cofactor biosynthesis pathway [9]. SAMP1 function in MPT biosynthesis is based on its structural homology to bacterial MoaD [12] and its indispensability in anaerobic DMSO respiration [9], which requires the molybdenum cofactor coordinating DMSO reductase.

While the role of SAMPs as sulfur carriers is associated with the biosynthesis of MPT and thiolated tRNA, the downstream consequences of sampylation are largely unknown. Sampylation is generally induced by DMSO, and SAMP2 conjugates accumulate in abundance upon addition of proteasome inhibitor [7]. However, unlike 20S proteasomes [13], the SAMP system is not essential for growth [7, 9]. Sampylation instead appears important to overcome stress [7, 9].

Among the three SAMPs identified to be protein modifiers (SAMP1/2/3) in *Haloferax volcanii* [7,8], the physiological contributions of sampylation are the least understood with only one protein target identified to date. The conserved K240 and K247 residues of MoaE, the large subunit of MPT synthase, are found to be covalently attached to C-terminus of SAMP1 [11]. In the modeled structure of MPT synthase, both target lysine residues are within the binding pocket of precursor Z [14]. Thus, SAMP1 conjugation is speculated to regulate the catalytic activity of MPT synthase.

Based on conjugate profiles, the number of SAMP1 targets is more extensive than MoaE suggesting a global proteomic approach would be useful in expanding knowledge of this system. However, unlike ubiquitin (-KGG), the C-terminal tail of SAMP1 (-SGG) is devoid of lysine and arginine residues. Thus, direct digestion of SAMP1 conjugates by trypsin is not

predicted to generate low-molecular-weight SAMP1-derived di-Gly remnants on lysine residues of target proteins that would be detected by MS.

Here amino acid residues isopeptide linked to SAMP1 were identified on a global scale. To enhance coverage, samp1ylated proteins were enriched from the archaeon *Hfx. volcanii* prior to analysis by LC-MS/MS. The SAMP1 S85R variant, found functional in sulfur mobilization and isopeptide linkage, was expressed in *Hfx. volcanii* strains (wild type, *ubaA* and *samp1-3*) and used to generate di-Gly footprints on target residues that were readily identified by MS. A total of 23 lysine residues mapping to 11 target proteins were identified under DMSO-inducing conditions. Many of the target proteins that were identified were associated with sulfur metabolism and oxidative stress pathways, with some of the target lysine residues predicted to be in close proximity to active site residues by 3D-homology modeling. Based on these results, we propose that samp1ylation regulates the activity of enzymes associated with oxidative stress and DMSO respiration in halophilic archaea such as *Haloferax volcanii*.

2 Materials and methods

2.1 Materials

All biochemical and analytical-grade inorganic chemicals were purchased from Fisher Scientific (Atlanta, GA, USA), Bio-Rad (Hercules, CA, USA), and Sigma-Aldrich (St. Louis, MO, USA). DNA polymerases and modifying enzymes were from New England Biolabs (Ipswich, MA, USA). Hi-Lo DNA standards were from Minnesota Molecular, Inc (Minneapolis, MN, USA). Taq DNA polymerase was from Bioline (Taunton, MA, USA). Desalted oligonucleotides were from Integrated DNA Technologies (Coralville, IA, USA).

2.2 Strains and growth conditions

Strains (including the primers and plasmids used to construct the strains) are summarized in Supporting Information Table 1. *Escherichia coli* Top10 was used for isolation of new plasmid constructs. *E. coli* GM2163 was used for replication of plasmid DNA prior to transformation into *Hfx. volcanii* according to standard methods [15]. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin (0.1 mg/mL) as needed. *Hfx. volcanii* strains were grown at 42°C in ATCC974 medium. Novobiocin (0.1 µg/mL) was included for growth of all *Hfx. volcanii* strains carrying pJAM202 derived plasmids. Cultures were supplemented with DMSO or DMF at 100 mM as indicated. For generation of deletion and integrant strains, *Hfx. volcanii* cells were plated on casamino acid medium with and without uracil and 5-fluoroorotic acid as previously described [16]. Media formulae were according to *The Halohandbook* [15]. Cells were grown in liquid medium with rotary shaking for aeration at 200 rpm and on solid medium using 1.5 % w/v agar plates. Cells were grown anaerobically on YPC medium with 100 mM DMSO as a terminal electron acceptor in 9-mL screw capped tubes (13 × 100 mm²). Growth was monitored by OD at 600 nm (OD₆₀₀).

2.3 Generation of mutant strains and site-directed mutagenesis

Mutant strains were generated by a *pyrE2*-based ‘pop-in/pop-out’ deletion method [16]. Deletion and integrant strains were identified by PCR using primer pairs annealing to the genome immediately flanking the genomic region carried on the plasmid used for homologous recombination. Amino acid exchange was performed by inverse PCR-based site-directed mutagenesis using Phusion DNA polymerase. DpnI-treated PCR amplicons were phosphorylated by T4 polynucleotide kinase and circularized by T4 DNA ligase prior to transformation into *E. coli* Top10. The DNA sequence fidelity of *trans*-expressed genes and PCR products derived from mutant strains was confirmed by Sanger automated DNA sequencing using an Applied Biosystems model 3130 genetic analyzer (ICBR Genomics Division, University of Florida).

2.4 Immunoblotting analysis

Protein samples were boiled for 10 to 15 min in SDS loading buffer [100 mM Tris-Cl, pH 6.8, with 2% w/v SDS, 10% w/v glycerol, 0.6 mg·mL⁻¹ bromophenol blue, and 2.5% w/v β-mercaptoethanol]. Proteins were separated by SDS-PAGE (10 or 12%) and electroblotted onto PVDF membranes (0.5 μm, Amersham). Equivalent protein loading was determined by OD₆₀₀ of whole cells, by bicinchoninic acid assay of protein concentration, and by Coomassie blue staining of parallel gels. Alkaline phosphatase (AP)-linked anti-Flag M2 monoclonal antibody (Sigma-Aldrich) and mouse anti-StrepII polyclonal antibody (Qiagen) combined with goat anti-mouse IgG (H + L)-AP-linked antibody (Sigma-Aldrich) were used for immunoblotting (IB). AP activity was detected by chemiluminescence with CDP-Star (Applied Biosystems) and X-ray film (Hyperfilm; Amersham Biosciences).

2.5 Protein concentration

Protein concentration was determined using the Bradford assay (BioRad) for screening chromatographic fractions and bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) for quantitative determination. BSA served as the protein standard (BioRad).

2.6 Purification of SAMP1 S85R conjugates

Hfx. volcanii strains expressing Flag-SAMP1 S85R were used for purification of SAMP conjugates. Strains for purification included H26 (wild type), HM1052 (*ubaA* E1-like mutant), and HM1096 (*samp1-3* triple mutant), with all three expressing Flag-SAMP1 S85R from plasmid pJAM556. *Hfx. volcanii* H26 carrying the empty vector (pJAM202c) was included as a control for nonspecific protein binding to the columns. Cells (1-L culture in 2.8 L Fernbach flask) were grown in ATCC974 medium supplemented with 100 mM DMSO with rotary shaking (200 rpm; 42°C). Cells were harvested at stationary phase (OD₆₀₀ of 2.5) by centrifugation (3500 × *g*, 10 min at 4°C) and washed in 15 mL buffer (50 mM Tris pH 7.5, 2 M NaCl). Cells were resuspended at 5 mL per g wet wt in high-salt lysis buffer (50 mM Tris-HCl, pH 7.4, 2 M NaCl, 1% Triton X-100, 1 mM EDTA) supplemented with nuclease (5 μg·mL⁻¹) and protease inhibitor cocktail (as directed by supplier, Sigma-Aldrich). Cells were lysed by thrice passage through a French pressure cell (20 000 psi). Lysate was clarified by centrifugation (13 000 × *g*, 25 min at 4°C) and filtration (0.45 μm filter; 25 mm surfactant-free cellulose acetate membrane, Nalgene). Cell-free extract (25–30

mg) was applied to an anti-Flag M2 column (1 cm dia. with 0.5 mL anti-Flag M2-affinity beads, Sigma-Aldrich) pre-equilibrated with TBS buffer (150 mM NaCl, 50 mM Tris-HCl, adjusted to pH 7.4). Bound proteins were washed with 20 column volumes of TBS and eluted with 0.1 mg 3× Flag peptide (Sigma-Aldrich) in 1 mL TBS. Protein fractions were pooled from the separately purified biological replicates (2 × 1-L cultures) for each of the three different *Hfx. volcanii* strains (wild type, *ubaA* and *samp1-3*) that expressed Flag-SAMP1 K85R *in trans*. Samples were desalted by dialysis (3.5-kDa cut-off) thrice against 4-L deionized water at 4°C and stored at -80°C until use.

2.7 MS-based mapping sites of samp1ylation

Protein fractions (25 µg per sample) purified by anti-Flag chromatography from each strain type were reduced, alkylated in-solution, and digested with trypsin (Promega) similarly to previously described [17]. In brief, enriched samp1ylated proteins (in 200 µL fractions) were treated with 5 µL of 200 mM DTT and incubated at 95°C for 5 min and 55°C for 30 min. To prevent alkylation of lysine residues, the reduced protein samples were alkylated with 2-chloroacetamide (4 µL of 2 M) in the dark for 45 min at room temperature and quenched with DTT (20 µL of 200 mM) at room temperature for 45 min. The enzymatically digested samples were injected onto a capillary trap (LC Packing C18 Pep Map nanoflow HPLC column) (EASY-nLC 1000 Proxeon, Thermo Scientific) and desalted for 5 min with a flow rate 300 nL/min of 0.1% v/v acetic acid. Peptide fragments were eluted by a linear gradient for 60 min at 300 nL/min starting at 3% solvent A and 97% solvent B, and finishing at 60% solvent A and 40% solvent B. Solvent A consisted of 0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H₂O. Solvent B consisted of 0.1% v/v acetic acid, 96.9% v/v ACN, and 3% v/v H₂O. MS/MS analyses of fractions were carried out on LTQ Orbitrap XL and Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometers (ThermoFisher Scientific). For the LQT Orbitrap XL, the ion spray voltage was set to 2200 V. Full MS scans were acquired with a resolution of 60 000 in the orbitrap from *m/z* 300–2000. The ten most intense ions were fragmented by CID. Dynamic exclusion was set to 60 s. For the Q Exactive Plus, a top 20 method was used. The ion spray voltage was set to 2180 V. Full MS scans were acquired with a resolution of 70 000 from *m/z* 400 to 2000. MS/MS scans were acquired with a resolution of 17 500. The 20 most intense ions were fragmented by high energy collisional dissociation (HCD). Dynamic exclusion was set to 60 s.

All MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0) with searches of the *Hfx. volcanii* DS2 [HaloferaxB_NCBI, 032511(March 25, 2011)] database assuming digestion with trypsin. FDR was specified at 0.1% using the automatic decoy database search in Mascot. Fragment ion mass tolerance was 0.8 Da, and parent ion tolerance was 10 ppm. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met, and -Gly-Gly signatures, were specified in Mascot as variable modifications. Scaffold (version Scaffold-4, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [18]. Protein identifications were accepted if established at greater than 95.0% probability and contained at least two identified unique peptides, as assigned by the Protein Prophet algorithm [19]. The four sample types (wt, *ubaA*,

samp1-3, and vector) were reproducibly isolated from *Hfx. volcanii* cells and analyzed by MS (twice by LTQ Orbitrap XL and twice by Q Exactive Plus hybrid quadrupole-Orbitrap MS) for a total of four replicates each.

2.8 Modeling of 3D protein structures

3D-structural models of *Hfx. volcanii* proteins were generated with high confidence (>90%) by combining 3D multitemplate-based alignment and computational folding simulations using Phyre2 (Protein Homology/AnalogY Recognition Engine) [20, 21]. NMR and crystal structures from the Structural Classification of Proteins (SCOP) and the Protein Data Bank (PDB) were used as fold templates. For dimeric complexes, UbaA was modeled (245 of 270 residues or 91% total) using the native *E. coli* MoeB-MoaD complex (PDB: 1JW9) and ThiF (PDB: 1ZFN) as fold templates. The remaining 25 residues of UbaA, which included the highly disordered N- and C-termini, were modeled by *ab initio*. The crystal structure of SAMP1 (PDB: 3PO0) [12] was docked to the 3D model of UbaA using the *E. coli* MoaD-MoeB complex as a template (PDB: 1JW9). MoaE-SAMP1 complex was modeled using *Staphylococcus aureus* MPT synthase bound to precursor Z (PDB: QIE2) as a scaffold.

2.9 Purification of UbaA, MoaE, and associated conjugates

UbaA and MoaE were purified from *Hfx. volcanii* strains expressing SAMP1 from its genomic locus. To monitor conjugate formation, UbaA and MoaE were expressed with C-terminal StrepII tags from plasmids pJAM957 and pJAM1119 in Flag-SAMP1 integrant strains YW1001 (wild type), YW1002 (*moaE*), and YW1004 (*ubaA*). Cells were grown with aeration to stationary phase (OD₆₀₀ ~2.5) in ATCC 974 medium with and without 100 mM DMSO (50 mL cultures in 250 mL flasks; rotary shaking at 200 rpm). Proteins were purified in high-salt buffer (50 mM Tris-HCl, pH 7.4, 2 M NaCl, 1 mM EDTA) supplemented with nuclease (5 µg/mL) and protease inhibitor cocktail (1.62 mg/mL, Sigma) by StrepTactin affinity chromatography as described previously [7].

3 Results

3.1 DMSO-inducible SAMP1 conjugates for global mapping

Here we find that samp1ylation extends beyond MoaE as a protein target. In particular, DMSO-treatment of aerobic cells was found to significantly increase the diversity of SAMP1 conjugates that could be detected by IB assay in wild type (Fig. 1A) and *moaE* mutant cells (Fig. 1B). These findings provided a rationale for use of global mapping to define new targets of samp1ylation.

3.2 Comparison of SAMP1 wild type and S85[R/K] variants

Prior to mapping the sites of samp1ylation on a global scale, SAMP1 S85[R/K] variants were compared to wild type for efficiency of proteome modification and ability to function in DMSO respiration. Like SUMO, the C-terminal tail of SAMP1 is devoid of basic residues. Thus, SAMP1 S85 [K/R] variants with basic residue substitutions immediately adjacent to the C-terminal diglycine were included in the analysis, since these SAMPs would generate diglycine remnants (114.1 Da) on target residues after trypsin digestion [11]. Efficiency of proteome modification was assessed by IB analysis of cell lysate separated by

reducing SDS-PAGE (Fig. 2A). *Hfx. volcanii* wild type and *ubaA* mutant strains were included to evaluate covalent linkages that required the E1-like UbaA. Conjugation of SAMP1 and its S85[K/R] variants was found to be dependent on the presence of *ubaA*, consistent with our earlier work that UbaA is required for sampylation [22]. When cells were grown aerobically in complex medium, the conjugation patterns of SAMP1 S85[K/R] variants were found similar to wild type (Fig. 2A). In particular, a single predominant protein band was detected at 53 kDa, which based on our earlier work corresponds to SAMP1 isopeptide-linked to MoaE [11]. Addition of DMSO to the culture medium increased the diversity of SAMP1 S85R conjugates in a banding pattern that was comparable to wild type but distinct from SAMP1 S85K (Fig. 2A). Additional conjugate bands of SAMP1 S85K were observed at 24, 35, and > 100 kDa suggested K85 served as a site of sampylation not found in wild type or increased the affinity of SAMP1 for use as a protein modifier. Further analysis of SAMP1 S85R revealed this variant could complement a *samp1* mutation for DMSO respiration similarly to SAMP1 wild type (Fig. 2B), suggesting the amino acid change did not alter the role of SAMP1 in sulfur transfer to form MPT. Thus, SAMP1 S85R was chosen as a tool to facilitate identification of SAMP1 conjugation sites, as a first step in understanding the physiological roles of sampylation on a global proteomic scale.

3.3 Mapping sampylation on a global proteomic scale

Conjugates of SAMP1 with the S85R amino acid exchange were readily purified from *Hfx. volcanii* cells grown under conditions found to stimulate sampylation (aerobically on rich medium supplemented with DMSO). In particular, both free and conjugated forms of SAMP1 S85R were isolated from H26 (parent, wild type) expressing the Ub-like protein, while only the free form of SAMP1 S85R was isolated from the *ubaA* mutant (Fig. 3). UbaA is required for sampylation [9]. Thus, the conjugates detected by this approach represented proteins with UbaA-dependent sites of sampylation.

To map the sites of sampylation, the purified fractions of *Hfx. volcanii* wild type, *ubaA* and *samp1-3* cells containing the free and/or conjugated forms of SAMP1 S85R were analyzed by trypsin-based LC-MS/MS. By this approach, 23 target lysine residues were mapped to 11 proteins in wild type and *samp1-3* cells (Table 1 and Supporting Information Table 2). No modified lysines were identified in the *ubaA* mutant. N-terminal methionine, serine, and cysteine residues were not found to be modified by SAMP1. A number of additional proteins that did not have target lysine residues identified, but were unique to the *ubaA*+ strains, were found to copurify with the sampylated proteins (Supporting Information Table 2). Whether these proteins had target sites that were not amenable to mapping by trypsin-based LC-MS/MS analysis or were in noncovalent complexes with the SAMP conjugates remains to be determined.

Many of the sampylated proteins that were identified (from cells grown aerobically on DMSO) were homologs of sulfur metabolism, oxidative stress, and/or autoregulation (Table 1). Proteins found multiply modified by sampylation are examples of this association including the E1-like UbaA, the ubiquitin-like SAMP3 (related to SAMP1), the large subunit of MPT synthase (MoaE), and a methionine sulfoxide-*S*-reductase homolog (MsrA).

Proteins with only single sites of sampylation identified included SAMP1, the Fe-S cluster assembly protein SufB, the methionine sulfoxide-*R*-reductase homolog (MsrB), translation initiation factor aIF-2 β , and proteins with less defined roles (isochorismatase family, cupin superfamily, and other conserved protein homologs). While MsrA and MsrB are not related in primary amino acid sequence, these enzymes have mirror-image active sites that commonly repair oxidized proteins by reducing methionine sulfoxide (S and R epimers, respectively) residues to methionine through formation of a sulfenic acid intermediate [23]. Previously, we found cells growing aerobically minus DMSO to also modify MoaE K240 and K247 by SAMP1 S85K [11] and SAMP3 A70K [7], and to modify UbaA K115 by SAMP2 [8]. Thus, in aerobic cells, UbaA and MoaE appear as major targets of sampylation irrespective of the presence of DMSO or type of SAMP modifier, suggesting tight autoregulation of UbaA-mediated SAMP activation and MPT synthesis. We also find here that upon treatment of cells with DMSO, additional proteins are conjugated to SAMP1 that are homologs of sulfur metabolism and oxidative stress pathways.

3.4 Ruling out samp2ylation

Working with the simple model system of *Hfx. volcanii* enabled us to conclude that many of the sampylated lysines were directly linked to SAMP1. Of the SAMPs encoded on the genome, only SAMP2 has a native (R/K) preceding the C-terminal di-Gly motif. Thus, the di-Gly footprints identified on 11 of the 23 lysines that mapped to four of the 11 target proteins when purified as SAMP1 S85R conjugates from a *samp1-3* triple mutant were concluded to be occupied by SAMP1 (see Table 1 for details). For the remaining sites that were found to be sampylated, it is possible that these lysines were directly modified by SAMP2 and were bound either covalently or noncovalently to the SAMP1 S85R bait. SAMP2 is readily detected using this type of LC-MS/MS analytical approach [8]; however, SAMP2 was not detected for any of the four replications of the four different sample types. Furthermore, our previous mapping of SAMP2 conjugation sites was to a separate set of target lysines with exception of K90 of the isochorismatase domain protein HVO_2328 and K113 of the E1-like UbaA [8], with the latter required for isopeptide linkage to all three SAMPs [7,9]. Reduced levels of the SAMP1 S85R fractions enriched from the *samp1-3* mutant could account for the inability to detect all target lysine residues when compared to wild type. Thus, at least half of the target lysines reported here are linked to SAMP1; the remaining are likely to be SAMP1, but novel linkages to SAMP2 cannot be ruled out.

3.5 Modeling sampylation sites on protein targets

To further understand the ubiquitin-like modifications that were identified, a Phyre2-based homology modeling approach was used to approximate the location of target lysine residues in relationship to overall protein structure. By this method, samp1ylation was predicted to inhibit MPT synthase activity through covalent modification of lysine residues located in the active site pocket of MoaE that binds precursor Z (K160, K240, and K247), the SAMP1 docking site of MoaE (K183 and K248), and the MoaE dimer interface (K139) (Fig. 4A). Likewise, sampylation was predicted to autoregulate the activity of UbaA based on finding one of the target lysine residues (K157) near the SAMP docking site (Fig. 4B). The influence of sampylation on UbaA K113 was less clear, as this residue was located on the surface of the SAMP-activating enzyme away from any residues predicted to be needed for

catalytic activity and/or subunit interfaces (Fig. 4B). The sites of MsrA/B that were modified by SAMP1 were also positioned at a significant distance from the catalytic active site including lysine residues within the unstructured C-terminal tail of MsrA (K169, K172, K180, and K182) and on the protein surface of MsrA (K108) and MsrB (K117) (Fig. 4C). While less is understood regarding SufB-mediated catalysis, the modified lysine (K350) of the *Hfx. volcanii* SufB homolog was predicted to reside on the protein surface at a distance from any cysteine residues that would serve as Fe-S assembly scaffolds (Fig. 4D). The SAMP3 K62 modified by sampylation was somewhat analogous to Ub K48 in its location on 3D structure as determined recently by NMR [24]. K62 was found at the carboxyl end of $\beta 3$, which is part of the β -grasp fold structure characteristic of Ub-like proteins (Fig. 4E). Although we cannot distinguish between SAMP3 covalent linkage to SAMP1 or SAMP2, finding SAMP3 modified at lysine residues reveals mixed chains of polySAMPs form in the cell. Overall, our modeling approach suggests sampylation can alter enzyme activity and/or docking sites for protein complex formation. While it is possible that SAMP1 targets proteins for degradation by proteasomes, our recent analysis of cells treated with the proteasome inhibitor bortezomib suggests that this role is primarily mediated by covalent linkage to SAMP2 and not SAMP1/3 [7].

3.6 Integration of Flag-SAMP1 onto the *Hfx. volcanii* genome

To further study sampylation, an N-terminal Flag-tag was integrated into the SAMP1 locus of the genome of *Hfx. volcanii* wild type and *ubaA* mutant strains. This approach allowed us to monitor the expression of SAMP1 from its native gene locus. The genomically encoded Flag-SAMP1 was found functional as a sulfur carrier protein based on the ability of the integrant strain to respire on DMSO (Fig. 2, columns 5–6). Likewise, expression of Flag-SAMP1 from its native promoter resulted in the formation of SAMP1 protein conjugates that were covalent, induced by DMSO, dependent on UbaA, and reduced by *trans*-expression of HvJAMM1 desampylase (Fig. 5). While use of the ectopic rRNA P2 promoter for SAMP1 *trans*-expression was beneficial for high throughput enrichment of SAMP1-conjugates, the Flag-SAMP1 integrant strain was preferred for the follow-up approaches based on its expression from the native promoter.

3.7 Targets of sampylation

Generation of the Flag-SAMP1 integrant allowed us to use a follow-up strategy to evaluate the validity of the SAMP1-conjugates identified by high-throughput MS/MS analysis. For this alternative approach, target proteins were *trans*-expressed with a C-terminal StrepII tag in the Flag-SAMP1 integrant, purified by StrepII affinity chromatography and assessed for covalent attachment to SAMP1 by IB analysis of the purified fractions. MoaE and UbaA were chosen among the target proteins for this analysis based on our earlier finding that the C-terminal StrepII tag does not perturb their function [9]. By this 'low-throughput' method, UbaA and MoaE were found to be covalently linked to SAMP1 expressed from its native gene locus (Fig. 5). Conjugates were detected by α -Flag IB of the StrepII pull down fractions with the signal confirmed to be specific for Flag-SAMP1 integrant strains expressing the StrepII tagged target protein compared to control strains (Fig. 5). Free forms of UbaA and MoaE were identified in the α -StrepII immunoblots by comparing their migration to the same proteins purified from recombinant *E. coli* (data not shown). The

identity of the PTM(s) responsible for the additional high molecular mass signals detected for UbaA and MoaE only by α -StrepII (and not α -Flag) IB remains to be determined. We speculate that these modifications are most likely the attachment of SAMP2/3 expressed from the genome, based on our previous finding that UbaA K113 is modified by SAMP2 and MoaE K240 and K247 are modified by SAMP3 [7, 8]. Differences in IB sensitivity of the α -StrepII and α -Flag antibodies may also be a reason. Nonetheless, our results provide strong evidence for the samp1ylation of UbaA and MoaE. Furthermore, we find that Flag-SAMP1 expressed from its native promoter can serve as an ubiquitin-like protein modifier even when the target protein is ectopically expressed from a plasmid.

4 Discussion

Here we provide an insight into understanding on how the archaeon *Hfx. volcanii* uses SAMP1 as a protein modifier to regulate and orchestrate cellular functions. Our multidisciplinary approach, which combines proteomics with genetics, reveals that SAMP1 modifies a relatively small number of protein targets and enables us to predict of how samp1ylation may impact protein function, through 3D homology modeling. A total of 11 proteins and 23 target lysines are identified based on detection of -Gly-Gly signatures using a high-throughput LC-MS/MS approach that relies upon site-directed mutagenesis and affinity tag enrichment prior to analysis. Protein targets include not only MoaE but also the E1-like UbaA, SAMP3, Fe-S assembly protein SufB, methionine-S-sulfoxide reductase (MsrA), and methionine-R-sulfoxide reductase (MsrB) with several of these proteins multiply modified by SAMP-linkages. 3D modeling of target lysines suggests samp1ylation occurs at or near active site residues of MoaE and UbaA that inhibit their activity, while heteromeric chain topologies are revealed by detected linkage of SAMP1/2 to SAMP3. Genetic approaches are found to provide complementary evidence that UbaA and MoaE are targets of samp1ylation with the added finding that SAMP1 expressed from its native promoter still serves as a sulfur carrier in DMSO respiration and an ubiquitin-like protein modifier, whose conjugation is stimulated by DMSO. Thus, we provide strong evidence that samp1ylation is a biologically relevant process that targets proteins associated with sulfur mobilization and oxidative stress.

Guided by these results, we propose a model in which samp1ylation regulates the transition from aerobic to anaerobic (DMSO) respiration in halophilic archaea such as *Hfx. volcanii*. Regulation of this metabolic transition would be an important advantage for survival in hypersaline lakes, which are abundant in algal-derived osmolytes such as dimethylsulphoniopropionate, a significant contributor to the global sulfur cycle and metabolic precursor of dimethylsulfide and DMSO [25,26]. During oxygenated nutrient-rich conditions, the large (MoaE) and small (SAMP1) subunits of MPT synthase are produced and appear to be maintained in an inactive form through samp1ylation. Consistent with this, *Hfx. volcanii* requires MPT biosynthesis for anaerobic (DMSO respiratory) not aerobic growth [9]. Transition to anaerobic conditions may stimulate the cleavage of the covalent SAMP1-MoaE linkages by isopeptidases such as HvJAMM1 [11]. Hydrolysis of these isopeptide bonds is exergonic and, thus, has the potential to reactivate MPT synthase without the need for energy input. Stimulation of this cleavage would, thus, activate MPT synthase and provide the MPT cofactor needed to coordinate the active site of terminal reductases

such as DMSO reductase and allow for anaerobic respiration during energy limited conditions. Archeal methionine sulfoxide reductases are soluble and, like yeast, may reduce DMSO to dimethyl sulfide [27]. If so, as the ratio of DMSO to oxygen rises, the observed samplation of MsrA/B could serve to inhibit these enzymes and maximize the availability of DMSO to serve as a terminal electron acceptor for energy production. In analogy to yeast [28], DMSO may also induce oxidative stress in *Hfx. volcanii*. Consistent with this, here we find that DMSO-induced the samplation of a discrete set of target proteins that were homologs in primary and/or predicted 3D structure to proteins associated with sulfur metabolism and oxidative stress (i.e., MsrA, MsrB, UbaA, SAMP3, and SufB). Samplation may regulate the stability and/or activity of these enzymes to ensure efficient proteome repair after exposure to oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP	alkaline phosphatase
IB	immunoblotting
MPT	molybdopterin
SAMP	small archeal modifier protein

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Significance of the study

This study provides a global perspective on ubiquitin-like protein modification systems of archaea by expanding knowledge of the sites of samp1ylation. We find that samp1ylation commonly targets proteins of sulfur metabolism and oxidative stress pathways including its isopeptide linkage to lysine residues of MoaE, UbaA, SufB, MsrA/B, and SAMP3. Reverse affinity purification was found useful in confirming selected substrates of samp1ylation. Phyre-based 3D protein homology modeling also helped guide understanding whether samp1ylation occurred on the protein surface or within the active site and suggested samp1ylation had an autoinhibitory role in modulating the activation of the SAMPs by the E1-like UbaA and the synthesis of molybdopterin. Finding two of the SAMP1 targets are linked to the reduction of DMSO (MsrA and MoaE, the latter through synthesis of the molybdopterin cofactor of DMSO reductase) suggests sampylated provides a balance—protecting against oxidative stress, yet ensuring DMSO is available for use in anaerobic respiration when oxygen levels become limiting.

Figure 1.

DMSO induces sampilylation of the *Hfx. volcanii* proteome. *Hfx. volcanii* parent (H26-pJAM947)(left panels, (A) and *moaE* mutant (HM1053-pJAM947) (right panels, (B) log-phase cells were inoculated at 0.1% v/v into ATCC 974 medium supplemented with DMSO, DMF, or no treatment as indicated. Cells were grown with aeration (rotary shaking, 200 rpm). Sampilylation was monitored over time by α -Flag immunoblotting analysis of proteins separated from whole cells (0.13 OD₆₀₀ units per lane) by reducing SDS-PAGE. Chemiluminescence detection of Flag-tagged proteins was by 3 h exposure of X-ray film.

Figure 2.

SAMP1 S85R does not influence the samp1ylome (A) or sulfur mobilization to molybdopterin (B). (A) Influence of SAMP1 S85 site-directed modifications on the samp1ylome determined by α -Flag immunoblotting analysis. *Hfx. volcanii* H26 parent and *ubaA* mutant strains expressing Flag-SAMP1 (wild type, S1), Flag-SAMP1 variants (S85K and S85R) and empty vector control (—) were grown in ATCC 974 medium with or without 100 mM DMSO as indicated. Proteins were separated by reducing SDS-PAGE (where H26 and *ubaA* strains were applied at an OD_{600} of 0.22–0.28 and 0.45 units of cells per lane,

respectively). Chemiluminescence detection of Flag-tagged proteins was by exposure of X-ray film for 45 min (upper panel) and 3–3.5 h (middle and lower panels). (B) Influence of SAMP1 S85R site-directed modification on sulfur mobilization to molybdopterin, as monitored by anaerobic growth using DMSO (100 mM) as a terminal electron acceptor for 96 h. *Hfx. volcanii* H26 parent (wild type, wt) and *samp1* mutant expressed Flag-SAMP1 wt and S85R *in trans* or from the *samp1* genomic locus are as indicated. Error bars represent SD of three biological replicates, with the results experimentally reproducible.

Figure 3. SAMP1 S85[R/K] conjugates purified from *Hfx. volcanii* H26 parent (wild type, wt) compared to a *ubaA* mutant (HM1052). Conjugates were purified as described in Section 2, separated by reducing SDS-PAGE (0.75–1.0 μ g protein per lane), and analyzed by α -Flag immunoblotting.

Figure 4.

Target sites of sampylation mapped to 3D protein structure as represented by ribbon diagram. (A) *Hfx. volcanii* MPT synthase complex (large and small subunits are in blue/green (MoaE dimer) and red ribbon (SAMP1), respectively). The C-terminal tail of SAMP1 (Ct), sites of sampylation (MoaE K139, K160, K183, K240, K247, and K248), and substrate (precursor Z) of MPT synthase are indicated. (B) *Hfx. volcanii* UbaA (E1-like SAMP activating enzyme, gray ribbon) and SAMP1 (red ribbon) complex. ATP, Zn²⁺ structural ion, and sites of sampylation (UbaA K113 and K157) are indicated. (C) *Hfx. volcanii*

methionine-sulfoxide reductase A and B (left and right, respectively; red ribbon) with sites of samlplation modeled to MsrA (K108, K169, and K172) and MsrB (K117) indicated. MsrA K180 and K182 were found to reside in the unstructured C-terminal tail and could not be modeled. The MsrA model was overlaid with the *Mycobacterium tuberculosis* MsrA (PDB: 1NWA, gray) crystal structure. Conserved active site cysteine residues of MsrA are indicated. RMS or (2S)-2-(acetylamino)-N-methyl-4-[(R)-methylsulfinyl]butanamide is shown bound to MsrB in structural analogy to *Xanthomonas campestris* MsrB (PDB:3HCI). (D) *Hfx. volcanii* SufB dimer (blue ribbons) with the lysine (K350) targeted for samlplation found positioned on the protein surface at a distance from conserved cysteine residues (yellow) which may serve as Fe-S scaffolds. (E) *Hfx. volcanii* SAMP3 NMR solution structure (PDB:2M19, blue) [24] compared to Ub crystal structure (PDB: 1UBQ, green) with sites of Ub-like/Ub conjugation (SAMP3 K62 and Ub K48) indicated. *Hfx. volcanii* MoaE, UbaA, MsrA/B, and SufB 3D structures were modeled as described in Section 2. PDB numbers are provided for the other structures.

Figure 5.

The proteome of *Hfx. volcanii* is found samp1ylated when cells express SAMP1 from its native genomic locus with an N-terminal Flag-tag. (A and B) UbaA-StrepII and MoaE-StrepII purified by StrepTactin affinity chromatography (IP: StrepII) from Flag-SAMP1 integrant strains. (C) HvJAMM1 desampylase expressed in Flag-SAMP1 integrant strains. Conjugate formation was analyzed by α -StrepII and α -Flag immunoblotting of reducing SDS-PAGE gels as indicated and described in Section 2. Nonspecific protein band detected in empty vector control (*).

Table 1Archeal ubiquitin-like modification sites identified under mildly oxidizing conditions^{a)}

Locus tag	Description	Lysine modified	Archea distribution
HVO_0558	UbaA: SAMP activating E1-like enzyme (EC: 2.7.7.-)	K113 ^{b)} *, K157 ^{b)} *	IPR000594, all phyla
HVO_0860	SufB: FeS assembly protein	K350 ^{b)} *	IPR010231, most phyla
HVO_2177	SAMP3: ubiquitin-like protein modifier	K18 ^{b)} , K55 ^{b)} , K62 ^{b)} *	IPR016155 (beta-grasp fold proteins), all phyla
HVO_2619	SAMP1: ubiquitin-like protein modifier	K4 ^{b)}	
HVO_1864	MoaE: molybdopterin synthase large subunit (EC 2.8.1.-)	K139, K160, K183, K240, K247, K248	IPR003448, most phyla
HVO_A0230	MsrA: Methionine-(S)-sulfoxide reductase (EC:1.8.4.11)	K108 ^{b)} , K169, K172, K180 ^{c)} , K182 ^{b)}	IPR002569, most phyla
HVO_2234	MsrB: Methionine-(R)-sulfoxide reductase (EC:1.8.4.12)	K117 I	IPR002579, most phyla
HVO_2328	Isochorismatase family protein	K90 ^{b)}	IPR000868, most phyla
HVO_0284	Cupin superfamily	K29 ^{c)}	IPR013096, most phyla
HVO_1611	Conserved protein	K45 ^{b)}	arCOG04616, haloarchaea
HVO_2242	Translation initiation factor aIF-2 β	K190 ^{b)} *	IPR016190, all phyla

^{a)} Ubiquitin-like modification sites were identified based on -Gly-Gly signatures detected by LC-MS/MS analysis at FDR of <0.1% and peptide threshold probability of > 96% (except for SAMP3 K62, which had a 93% peptide probability score). Protein threshold of >99.0% probability was used in protein identification. All cells were treated with DMSO during growth. Flag-SAMP1 S85R protein conjugates were purified from wild type and *samp1-3* triple mutant strains. Flag-SAMP1 S83R purified from the E1-like *ubaA* mutant served as a control for isopeptide bond formation, while wild type cells carrying the empty vector served as a control for nonspecific protein copurification.

All -Gly-Gly-modified lysines were reproducibly identified unless otherwise indicated with an asterisk (*).

Tandem mass spectrometry (MS/MS) spectra, probability scores and other supporting data are available as supplementary materials (Supporting Information Table 2 and Fig. 1). Similarly to ubiquitin [29], trypsin was found to cleave after lysine residues modified by the ubiquitin-like isopeptide bond.

^{b)} Gly-Gly-modified lysines identified only in wild type strain expressing Flag-SAMP1 S85R.

^{c)} Gly-Gly-modified lysines identified only in *samp1-3* triple mutant strain expressing Flag-SAMP1 S85R.