

***Haloferax volcanii* UbaA, catalytic engine for sanylation and sulfur transfer**

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At first sight, the modification of proteins by ubiquitin-like proteins (Ubls) and the mobilization of sulfur into small molecules and tRNA do not have much in common. However, research in the last decade has made it clear that these enzymatic pathways are mechanistically and evolutionarily related [1]. The Small Archaeal Modifier Proteins (SAMPs) from *Haloferax volcanii*, which belong to the family of Ubl proteins, provide some of the clearest illustrations to date for this link [2], because the same Ubls act as protein modifiers [3] and are involved in sulfur transfer [4]. SAMP1, a member of the MoaD clade of Ubls [5], acts as a covalent protein modifier for UbaA, MoaE, and a set of other substrates [6]. At the same time, SAMP1 is required for molybdenum cofactor biosynthesis and therefore for anaerobic respiration with DMSO as the final electron acceptor [4]. SAMP2, a member of the ThiS clade of Ubls [5], can also be conjugated to some proteins [3] but it is also involved in tRNA thiolation [4]. In the context of proteasome deficiency (and also in the absence of an Rpn11-related isopeptidase), SAMP2 conjugate levels increase, suggesting that SAMP2 targets proteins for degradation [3], as indeed confirmed recently for at least one substrate [7]. Contrary to an initial report, SAMP3 also turned out to modify other proteins after correction of a genome annotation error [8]. Whether SAMP3 has a role in sulfur mobilization is not yet clear.

At the heart of the link between protein modification and sulfur transfer lies shared chemistry of the first step of the reactions [1]. Both start with the conversion of the SAMPs to the corresponding adenylates, at the expense of ATP, and with concomitant production of pyrophosphate (Fig. 1A) [9]. From there on, the pathways for protein conjugation and sulfur mobilization diverge. For sanylation, a SAMP adenylate is converted by cysteine (thiolate) nucleophilic attack to a thioester, which is subsequently resolved by nucleophilic attack of an amine (of a lysine residue in a protein

substrate), without intervening transthioesterification steps (Fig. 1B). Sulfur mobilization is thought to involve attack of a persulfide (from a rhodanese domain) to generate an acyl-disulfide, which is resolved to the thiocarboxylate by disulfide exchange (Fig. 1C). Transfer of the SAMP thiocarboxylate sulfur to its final destination is then catalyzed by downstream enzymes. The catalytic engine for SAMP activation is UbaA [3]. In the accompanying publication, J. Maupin-Furlow and colleagues provide a thorough biochemical characterization of UbaA and a detailed comparison with other related enzymes involved in either sulfur transfer or protein conjugation [10].

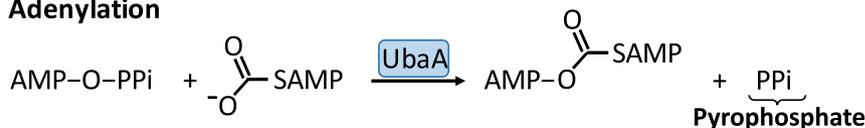
The UbaA sequence shows strong similarities to the sequences of *E. coli* MoeB and ThiF, as well as to yeast Uba4p and its mammalian ortholog MOCS3 (the urmylation E1). The region of similarity comprises the core adenylation domain, which adopts a variant of the canonical Rossmann fold [11]. In particular, the glycine-rich loop (reminiscent of the P-loop of other ATPases), two CXXC motifs, predicted to bind a structural zinc ion, and the 'active' cysteine are conserved. Like *E. coli* MoeB and ThiF, and unlike Uba4p and MOCS3, UbaA lacks a rhodanese domain, which is likely provided in *trans* [4]. Like other homologs, UbaA is expected to, and indeed does, form dimers.

Hepowitz and colleagues begin their characterization of UbaA with a check of the stabilizing effects of various nucleotides on UbaA. As expected, the natural substrate ATP and its analog AMP-PNP (with nonhydrolyzable β - γ , not α - β phosphate bond) stabilize UbaA most. The authors then proceed to check binding of SAMP1 and SAMP2 by isothermal scanning calorimetry (ITC). As expected, the presence of ATP enhances binding, to surprisingly varying degrees. The effect is slight for SAMP1, whereas ATP is strictly required for SAMP2 binding. SAMP1 and SAMP2 are quite dissimilar (only 21% identity). Nevertheless, it comes as a surprise that SAMP1 binding is

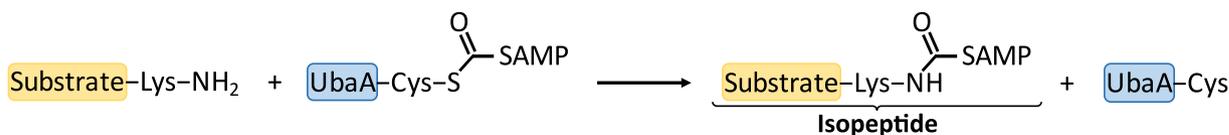
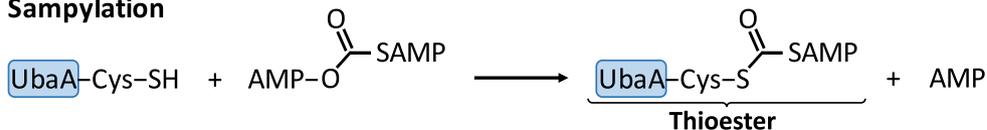
Abbreviations

DMSO, dimethyl sulfoxide; ITC, isothermal scanning calorimetry; SAMPs, small archaeal modifier proteins; Ubls, ubiquitin-like proteins.

A Adenylation



B Sampylation



C Sulfur transfer

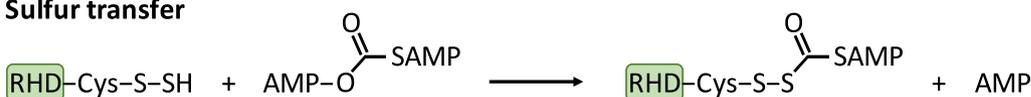


Fig. 1. The first steps of protein modification by UbIs and sulfur transfer are chemically identical. They begin by the ATP-dependent conversion of the SAMPs to the corresponding adenylates, with concomitant production of pyrophosphate (A). Highly speculative mechanistic model for the role of UbaA in sampylation (B) and sulfur transfer (C) based on an analogous model for the role of Uba4p [1]. The persulfide carrier, probably a rhodanese domain protein, remains to be identified. *In vivo*, disulfide-linked complexes of UbaA and a rhodanese domain protein would not be stable in the reducing intracellular environment. Note that competing, and only qualitatively similar mechanisms for sulfur transfer have been suggested for related systems (e.g., ThiF-ThiS), and that it is not yet clear that the mechanism adapted from Uba4p-Urm1 is the most appropriate one for sulfur transfer by the UbaA-SAMPs.

entropically and SAMP2 binding enthalpically driven, leaving the reader wondering whether binding modes are really as similar as homology modeling implies. Next, Hepowit and colleagues monitor noncovalent complex formation of UbaA and SAMPs *in vivo*. Interestingly, lesser amounts of SAMP1 and SAMP2 are pulled down with UbaA in the presence of DMSO. For SAMP1, this may reflect more efficient downstream use of the adenylates. For both SAMP1 and SAMP2, it additionally suggests, in agreement with earlier work [3], that sampylation is not a house-keeping activity, but is highly dependent on environmental conditions.

Based on the crystal structures of the MoeB-MoaD [12] and ThiF-ThiS [13], Hepowit and colleagues build a model of UbaA. The model is very confident around the ATP-binding site, and also in the region of the structural zinc ion. The ‘active’ cysteine (C188) is also unambiguously identified, and mapped to an ‘unstructured’ region presumably corresponding to the ‘cross-over loop’, thought to be held in place by the

structural zinc ion [11]. The authors validate their model by site-directed mutagenesis. Protein variants are tested for their ability to replace the wild-type protein for sampylation, anaerobic growth of *H. volcanii* with DMSO as a terminal electron acceptor (testing sulfur transfer to the molybdenum cofactor) and sulfur transfer to tRNA^{Lys}_{UUU}. Mutations designed to affect nucleotide binding or SAMP adenylation are expected to affect all UbaA activities. For some amino acid exchanges (K87R and D131N) this is indeed observed. However, at least one mutation (R74Q) affects sampylation more severely than sulfur transfer. Perhaps sulfur transfer copes better with reduced UbaA activity. Alternatively, R74 could have an unforeseen and still ill-understood role in sampylation.

Next, Hepowit and colleagues probe the role of tetrad cysteine residues of UbaA, using the same assays. The first (C171 and C245), but not the second (C174 and C248) cysteine residues of the CXXC motifs turn out to be essential in all assays. The requirement for some, but not other zinc ion ligands is surprising. In

MoeB, all four equivalent cysteine residues are required for sulfur transfer [14]. Surprisingly, mutation of the 'active site' cysteine (C188) did not completely abolish sumpylation, but did block sulfur transfer completely. This result questions the identification of the 'active site' cysteine with the catalytic cysteines of the E1s for ubiquitin, NEDD8, and SUMO. Indeed the latter cysteines are embedded in catalytic cysteine domains that have no counterpart in UbaA. C265 appears important for tRNA thiolation only. Hepowit and colleagues consider the possibility that this cysteine may be involved in disulfide exchange to convert SAMP acyl disulfides into thiocarboxylates. The idea is appealing, but it remains unclear why C265 is then not required for anaerobic growth with DMSO as the final electron acceptor. Therefore, we prefer the model that the 'active site' cysteine (C188) plays this role.

In vitro data for SAMP-UbaA complex formation are largely in agreement with expectations based on prior chemical knowledge and the *in vivo* data. As SAMP adenylation is a required step, SAMP-UbaA thioester formation is ATP-dependent. Surprisingly, AMP-PNP (with nitrogen replacing oxygen between β - and γ -phosphate) cannot substitute for ATP, making the reader wonder whether adenylation really involves a split between α - and β -phosphate and the production of pyrophosphate. Any doubts, however, are eliminated by reference to earlier work [9] and the observation that AMP-PNP is a poor substrate of ubiquitin-E1 too [15]. Consistent with the chemistry of thioesters, the covalent SAMP UbaA complexes are (mostly) sensitive to reducing agents, and their formation can be altogether prevented by pretreatment of UbaA with N-ethylmaleimide, a chemical that covalently modifies cysteine residues. In the *in vitro* assay, the 'active site' cysteine (C188), partially required according to the *in vivo* assay, is altogether not required, suggesting that other UbaA cysteines, in *cis* or *trans*, are better positioned to resolve SAMP adenylates.

UbaA is unusual among E1-like enzymes in being stably modified by its own substrates (through isopeptide, not thioester linkage). This has previously already been reported for SAMP1 and SAMP2 [3,6]. Here, the authors show that UbaA can be autosampylated by all three SAMPs, and they identify a new site for sampylation (K87). Finally, Hepowit and colleagues also show that UbaA autosampylation by all three SAMPs can be reversed by HvJamm1, an Rpn11-related isopeptidase [16].

In summary, Hepowit and colleagues provide the most detailed characterization so far of UbaA, the catalytic engine of the sampylation system of the archaeon *H. volcanii*. Dual-purpose E1-Ubl systems

are also known from eubacteria (TtuC/TtuB) and eukaryotes (Uba4p/Urm1) [2]. The present data are particularly interesting in comparison with results for those systems (see Table 3 of Hepowit *et al.* [10]). Together, they shed light on the evolution of E1-Ubl systems from more ancient sulfur transfer machinery.

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