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Structure–function analysis of Sua5 protein reveals novel functional motifs required for the biosynthesis of the universal t⁶A tRNA modification

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ABSTRACT
N⁶-threonyl-carbamoyl adenosine (t⁶A) is a universal tRNA modification found at position 37, next to the anticodon, in almost all tRNAs decoding ANN codons (where N = A, U, G, or C). t⁶A stabilizes the codon–anticodon interaction and hence promotes translation fidelity. The first step of the biosynthesis of t⁶A, the production of threonyl-carbamoyl adenylate (TC-AMP), is catalyzed by the Sua5/TsaC family of enzymes. While TsaC is a single domain protein, Sua5 enzymes are composed of the TsaC-like domain, a linker and an extra domain called SUA5 of unknown function. In the present study, we report structure–function analysis of Pyrococcus abyssi Sua5 (Pa-Sua5). Crystallographic data revealed binding sites for bicarbonate substrate and pyrophosphate product. The linker of Pa-Sua5 forms a loop structure that folds into the active site gorge and closes it. Using structure-guided mutational analysis, we established that the conserved sequence motifs in the linker and the domain–domain interface are essential for the function of Pa-Sua5. We propose that the linker participates actively in the biosynthesis of TC-AMP by binding to ATP/PPi and by stabilizing the N-carboxy-L-threonine intermediate. Hence, TsaC orthologs which lack such a linker and SUA5 domain use a different mechanism for TC-AMP synthesis.

Keywords: tRNA modification; threonylcarbamoyl adenosine; t⁶A₃⁷; Sua5; TsaC

INTRODUCTION
To be functional in the cell, tRNA molecules need to be post-transcriptionally modified by the addition of a variety of chemical groups (Grosjean et al. 1995). One of the most complex modifications is the N⁶-threonyl-carbamoyl adenosine (t⁶A) found exclusively at position 37 (3’ adjacent to anticodon) of tRNAs that decode codons of the type ANN (where N = A, U, G, or C) (Fig. 1A; Chheda et al. 1969; Takemura et al. 1969; Powers and Peterkofsky 1972). Except in some insect symbionts with highly reduced genomes, the t⁶A modification is ubiquitously distributed among cellular organisms as well as in organelles such as mitochondria and chloroplasts (Machnicka et al. 2013; Boccaletto et al. 2018). t⁶A prevents framenoshifting during translation by stabilizing the pairing between the A₃ from the codon and the U₃₆ from the anticodon (Weissenbach and Grosjean 1981; Sonawane and Sambhare 2015). In addition, its presence was reported to be critical for the binding of the anticodon stem–loop to the mRNA-ribosome complex in vitro (Stuart et al. 2000; Yarian et al. 2002; Murphy et al. 2004; Lescrinier et al. 2006; Agris 2008). Abolition of t⁶A biosynthesis is lethal in Escherichia coli (E. coli) (El Yacoubi et al. 2009; Thiaville et al. 2015a). In Saccharomyces cerevisiae (S. cerevisiae), the absence of t⁶A increases the occurrence of frameshifting, leaky scanning of start codons, and read-through of stop codons and leads to a dramatically reduced fitness (Lin et al. 2010; Daugeron et al. 2011; El Yacoubi et al. 2011; Thiaville et al. 2016). Recently, mutations in the
t6A synthetase genes were linked to a severe neurological and renal genetic disease in humans (Braun et al. 2017; Edvardson et al. 2017).

The t6A biosynthesis reaction proceeds in two consecutive steps (Fig. 1B) catalyzed by two universal enzyme families: Sua5/TsaC∗ (∗, previously YrdC) and Kae1/TsaD‡ (‡, previously YgjD). Sua5/TsaC catalyzes the condensation of L-threonine, bicarbonate (HCO$_3^-$) or carbon dioxide (CO$_2$), and ATP to form inorganic pyrophosphate (PPi) and L-threonyl-carbamoyl adenylate (TC-AMP). The exact nature of the carbonaceous substrate, HCO$_3^-$ or CO$_2$, is not yet unambiguously identified. The TC-AMP intermediate is taken up by Kae1/TsaD/Qri7 that transfers the L-threonyl-carbamoyl moiety onto the substrate tRNA to form t6A-tRNA (El Yacoubi et al. 2009, 2011; Srinivasan et al. 2011; Lauhon 2012; Deutsch et al. 2012; Perrochia et al. 2013a; Wan et al. 2013). Except in mitochondria, where Qri7 works alone as a homodimer (Wan et al. 2013; Thiaville et al. 2014), Kae1/TsaD requires the presence of essential accessory proteins to be active. In bacteria, TsaD forms with two bacteria-specific proteins, TsaB (previously YeaZ) and TsaE (previously YjeE), a complex called DEZ (Deutsch et al. 2012; Lauhon 2012; Nichols et al. 2013; Zhang et al. 2015b). In archaea and in the cytoplasm of eukaryotes, Kae1 associates with Bud32, Pcc1, and Cgi121 into a complex called KEOPS or EKC (recently renamed TCTC [for nomenclature of t6A synthetic genes, see Thiaville et al. 2014]), hereafter referred to as KEOPS for simplicity (Downey et al. 2006; Kisseleva-Romanova et al. 2006; Mao et al. 2008; Perrochia et al. 2013b; Thiaville et al. 2014; Zhang et al. 2015b). A fifth member of KEOPS, Gon7, is found exclusively in fungi and its counterpart, C14ORF142, was recently reported in humans and several other metazoans (Zhang et al. 2015a; Wan et al. 2016). The exact function of the accessory proteins of both DEZ and KEOPS complexes is unknown.

The TsaC protein from *E. coli* (Ec-TsaC) folds in a compact α/β twisted open-sheet structure composed of seven α-helices and seven adjacent β-strands in parallel and antiparallel orientations (Teplova et al. 2000; Fu et al. 2010; Harris et al. 2015). The overall structure is reminiscent of a baseball glove with a large central concave cavity. This cavity has a positive global charge and was suggested to act as nucleic acid binding surface (Teplova et al. 2000). Indeed, Ec-TsaC protein binds tRNA or the anticodon arm structure with high affinity in
vitro (El Yacoubi et al. 2009; Harris et al. 2011, 2013). The active site of Sua5/TsaC is found in this cavity and involves several highly conserved residues among which is the tetrad K^{28}xR^{52}/S^{139}xN^{141} (Ec-TsaC numbering) that plays a role in the binding of ATP and is essential for activity both in TsaC and Sua5 proteins (Teplova et al. 2000; El Yacoubi et al. 2009; Kuratani et al. 2011; Wan et al. 2013; Harris et al. 2015). The C-terminal extremity of the Ec-TsaC protein was shown to have an abnormally high flexibility and was proposed to be important for the enzymatic activity by acting like a gating loop (Harris et al. 2015).

Sua5 protein comprises an N-terminal TsaC-like domain and a C-terminal SUA5 domain. The two domains are connected via a linker containing about 40 residues (Fig. 1C). The structure of Sulfolobus tokodaii Sua5 (St-Sua5) was resolved in complexes with L-threonine and AMP-NPP (Kuratani et al. 2011) and with TC-AMP (Agari et al. 2008; Parthier et al. 2012). The overall fold of the TsaC-like domain of St-Sua5 is very similar to Ec-TsaC while the SUA5 domain adopts a Rossmann fold (Agari et al. 2008; Kuratani et al. 2011). However, about twenty residues in the linker connecting the two domains were not resolved in the structures of Sr-Sua5.

Despite the available atomic structures of Sua5 and TsaC enzymes, the mechanism by which the Sua5/TsaC catalyze the formation of TC-AMP is not yet understood. TC-AMP is an unstable molecule with a half-life of 3.5 min under standard conditions, i.e., pH 7.5, 37°C, 2 mM MgCl₂ (Lauhon 2012). Decomposition of TC-AMP in aqueous media produce AMP and a nearly equimolar mixture of L-threonine and its cyclic derivate 5-methyl-2-oxazolidinone-4-carboxylate as final products (Lauhon 2012). Despite this instability, direct contact between Sua5 and Qri7 from S. cerevisiae is not required for in vitro t²A synthesis, suggesting that TC-AMP is a diffusible reaction intermediate (Wan et al. 2013). Still, the exact mechanism of transfer of TC-AMP during t²A synthesis remains to be fully elucidated. Domains homolog to TsaC are also found in the (NiFe)-hydrogenase maturation factor HypF and the S. cerevisiae His67, Arg121, Ser181, and Arg195 (Supplemental Fig. S2B). It forms hydrogen bonds (H-bonds) with five highly conserved residues of the catalytic domain: Thr35, His67, Arg121, Ser181, and Arg195 (Supplemental Fig. S2B). A residual electron density cloud at the bottom of the catalytic pocket could be fitted by a L-threonine molecule, which copurified with the enzyme. L-threonine occupies the same pocket as the threonyl moiety of TC-AMP in the St-Sua5 structure (Supplemental Fig. S2B,C). It forms hydrogen bonds (H-bonds) with five highly conserved residues of the catalytic domain: Thr²⁵, His²⁷, Arg¹²¹, Ser¹⁸¹, and Arg¹⁹⁵ (Supplemental Fig. S2B). Atothe active site, two H₂O molecules (Supplemental Fig. S3B) are bound tightly via H-bonds with the conserved residues Arg⁵⁸, Asn⁶⁴, and His²³⁴ for one and Arg⁵⁸ and Ser¹⁴⁷ for the other.

The main difference between the structures of Pa-Sua5 and of Sr-Sua5 resides in the linker peptide connecting the TsaC-like and the SUA5 domains (Supplemental Fig. S2A). This region is partly disordered in the St-Sua5 structures, respectively from Pro²¹⁵ to Lys²³³ (St-Sua5 numbering) for the TC-AMP bound form (PDB: 4E1B) and from Glu²¹⁶ to Lys²³⁶ for the AMP-NPP bound form (PDB: 3AJE). In Pa-Sua5, the linker is fully ordered (Fig. 2A), starting with one short α-helical turn from Pro²¹⁵ to Tyr²³⁸ followed by a twisted hairpin from Asp²²³ to Lys²³¹ that enters the active site pocket and continues by a ω-loop that folds up against the active site cavity before joining the SUA5 domain. This

RESULTS

Crystallographic structure of Sua5 from P. abyssi reveals a novel conformation of the linker

We first determined the apo-structure of Pa-Sua5 at 2.8 Å resolution. The asymmetric unit contains four copies of Pa-Sua5 with an average root mean square deviation (RMSD) of 0.6 Å over 341 Ca atoms. The molecular mass of Pa-Sua5 in solution, as determined by SEC-MALLS is 38.02 kDa, which is consistent with Pa-Sua5 being a monomer (Supplemental Fig. S1) as reported for St-Sua5 (Agari et al. 2008; Kuratani et al. 2011). Pa-Sua5 and Sr-Sua5 share 80% sequence similarity and their overall structure is very similar, with an RMSD of 0.67 Å over 318 Ca atoms (Supplemental Fig. S2A). A residual electron density cloud at the bottom of the catalytic pocket could be fitted by a L-threonine molecule, which copurified with the enzyme. L-threonine occupies the same pocket as the threonyl moiety of TC-AMP in the St-Sua5 structure (Supplemental Fig. S2B,C). It forms hydrogen bonds (H-bonds) with five highly conserved residues of the catalytic domain: Thr²⁵, His²⁷, Arg¹²¹, Ser¹⁸¹, and Arg¹⁹⁵ (Supplemental Fig. S2B). Atothe active site, two H₂O molecules (Supplemental Fig. S3B) are bound tightly via H-bonds with the conserved residues Arg⁵⁸, Asn⁶⁴, and His²³⁴ for one and Arg⁵⁸ and Ser¹⁴⁷ for the other.

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linker conformation is stabilized by several interactions involving conserved residues (Fig. 2B). The α-helical turn is stabilized through H-bonds between the backbone of Ala216 and Val217 and the amine functions of, respectively, Lys331 and Asn327 in the SUA5 domain. The hairpin is stabilized through interaction between Lys58 and the residue Thr187 in the TsaC-like domain. Lys231 forms H-bonds with Asp61 in the TsaC-like domain and with the backbone of Asp226. Finally, Tyr232 forms H-bonds with Asp61.

To map the substrate/product binding sites of Pa-Sua5, we co-crystallized the protein with ATP, L-threonine, HCO3− and MgCl2. Crystals were obtained in the same conditions as for the L-threonine bound form and diffracted at 2.6 Å. The asymmetric unit contains four copies of Pa-Sua5 with an average RMSD of 0.53 Å over 340 Ca atoms. There was no indication for bound nucleotides, but we observed residual electron density in the active site cavity, which could be satisfactorily fitted by a Pi ion and an L-threonine (Fig. 2A). L-threonine occupies the same position in both Pa-Sua5 structures (Supplemental Figs. S3A, S4CD). Pi was likely generated by the hydrolysis of ATP during the crystallization and displaces two water molecules present in the L-threonine bound structure (Supplemental Fig. S3C). Pi is bound at the periphery of the active site where it occupies a positively charged electrostatic surface potential patch (Supplemental Fig. S3D). The oxygens of the phospho-moiety are coordinated by H-bonds respectively with Arg58, Asn62, and Gly229 and with Lys56, Arg58, Ser147, Gly148, and His234 (Supplemental Fig. S3C). These are all highly conserved residues and Gly229 and His234 belong to the linker (Supplemental Fig. S3C). This is a distinctive feature of Sua5 proteins because the TsaC proteins have no linker. Lys56, Arg58, and Ser143 belong to the conserved tetrad K50XR58/S143XN145 (Pa-Sua5 numbering), shown to be essential for ATP binding. Pi does not occupy exactly the same position as the β- and γ-phosphate groups of AMPPNP in St-Sua5 (Supplemental Fig. S4B,D), but it clashes with the β-phosphate of AMPPNP and with the phospho-moiety of TC-AMP (Fig. 3C). This suggests that the presence of Pi in the active site of Pa-Sua5 may compete with binding of a nucleotide. It was shown that YrdC is capable of catalyzing the reverse reaction at high concentrations of Pi (Lauhon 2012). The phospho-moiety of TC-AMP is oriented in a different direction compared to the β- and γ-phosphate groups of AMPPNP, and Pi is ideally positioned for the reverse nucleophilic attack of the phosphate of TC-AMP, yielding ATP.

The linker is superposable in both Pa-Sua5 structures, indicating that Pi is not required for its structuration (Supplemental Fig. S3A). The linker hangs over the active site gorge, blocking its access (Fig. 3A). The adenylate parts of AMPPNP and TC-AMP occupy the same position in the St-Sua5 structures (Supplemental Fig. S4A,B). Structure superposition of Pa-Sua5 with St-Sua5 reveals a clash between the linker hairpin residues Lys226-Ala227-Pro228 and the adenyl-moiety of both AMPPNP and TC-AMP (Fig. 3C). This conformation of the linker is therefore not compatible with the presence of a nucleotide in the active site. In Pa-Sua5, the conserved His234 of the linker binds to Pi while...
in the TC-AMP bound form of St-Sua5, its side chain points toward the solvent (Supplemental Fig. S4). Similarly, the side chain of the neighboring residue Tyr$^{239}$ is oriented deeper in the active site in the AMPPNP St-Sua5 structure, forming a H-bond with the β phosphate of AMPPNP and the Ser$^{144}$ of the KxR/SxN tetrad (Supplemental Fig. S4B). The binding of ATP and/or TC-AMP may thus be regulated via these two linker residues. A highly conserved proline, Pro$^{337}$, is found next to the bend of the linker C-terminal extremity (Supplemental Fig. S4).

The Pa-Sua5 domain adopts a Rossmann fold with an inner β sheet composed of five strands and framed by three α helices. Helices α10 (Val$^{289}$ to Arg$^{305}$) and α11 (Gly$^{320}$ to Ser$^{335}$) form a typical α/α coil, interacting via a hydrophobic zinc zipper formed by the conserved Leu$^{296}$, Leu$^{300}$, Val$^{323}$, and Leu$^{329}$. Several conserved residues of α10 and α11 form a network of noncovalent interactions with residues from the catalytic domain conserved among Sua5 proteins. Notably, Arg$^{301}$ forms H-bonds with the side-chain of Asp$^{161}$ and with the Pro$^{300}$ and Pro$^{152}$ backbones and, in a similar way, Arg$^{328}$ interacts with Glu$^{180}$ and Pro$^{196}$ (Fig. 2C). Arg$^{328}$ are like two arms holding together the two domains of Sua5-proteins.

**In vitro kinetics of ATP hydrolysis by Pa-Sua5wt**

Sua5/TsaC proteins require l-threonine, ATP, and HCO$_3^-$/CO$_2$ to synthesize TC-AMP and PPi via a complex mechanism that involves the formation of two covalent bonds (Agari et al. 2008; El Yacoubi et al. 2009; Lauhon 2012; Harris et al. 2015). The precise reaction mechanism of the enzymes is still unknown. As an initial step toward the elucidation of the catalytic mechanism of Pa-Sua5$^{wt}$, we determined the kinetic constants for the hydrolysis reaction of ATP into AMP. We used a radioactive assay to measure the initial velocity of the reaction at varying concentrations of either l-threonine or ATP. The concentration of HCO$_3^-$/CO$_2$ was constant as it is always present in solution if no precautions are undertaken. The best nonlinear least squares fit of the data produced a hyperbolic curve for both substrates, indicating that the reaction obeys the classical Michaelis–Menten kinetics (Supplemental Fig. S5). We found $K_m$ and $k_{cat}$ values of 11.5 ± 1.3 μM and 0.054 ± 0.005 sec$^{-1}$ for l-threonine, and 5.5 ± 1.3 μM and 0.029 ± 0.001 sec$^{-1}$ for ATP. The unexpected difference in $k_{cat}$ values may be a consequence of large variations in quantification of radioactive AMP at low ATP concentrations (Supplemental Fig. S5B). Our data are comparable to the apparent rate constants for the ATPase activity of St-Sua5 of 0.04 and 0.1 sec$^{-1}$ at 37 and 60°C, respectively, in the presence of 1 mM ATP, 5 mM MgCl$_2$, 50 mM K-HEPES pH 7.5, and 100 mM KCl (Agari et al. 2008).

The TsaC-like domain of Pa-Sua5 is highly unstable in the absence of the SUA5 domain

The structures of the catalytic domains of Ec-TsaC and of Pa- and St-Sua5 enzymes are very similar. To find out if the SUA5 domain is required for TC-AMP synthesis, we tested truncated variants of Pa-Sua5. We made three constructs: Pa-Sua5Δ$^{240–340}$ and Pa-Sua5Δ$^{200–340}$ correspond to the TsaC-like domain, respectively with or without the linker; Pa-Sua5Δ$^{1–239}$ corresponds to the SUA5 domain. After expression in E. coli, Pa-Sua5Δ$^{200–340}$ and Pa-Sua5Δ$^{240–340}$ could not be detected by Coomassie staining in the soluble fraction, and Pa-Sua5Δ$^{1–239}$ was significantly less soluble.
than Pa-Sua5\textsuperscript{wt} under different expression conditions tested (see Materials and Methods). Still, small amounts of Pa-Sua5\textsuperscript{Δ(200–340)}, Pa-Sua5\textsuperscript{Δ(240–340)}, and Pa-Sua5\textsuperscript{Δ(1–239)} could be obtained in soluble and pure form but none of them exhibited detectable ATPase activity in vitro (data not shown). We noticed that these truncated proteins were rapidly degraded under several storage conditions tested, indicating that they are highly unstable. Linker and SUA5 domain thus appear to be essential for the stability of the Tsac-like domain of Pa-Sua5.

**Mutations in the conserved residues of the linker and SUA5 domain produce stable proteins**

The structure of Pa-Sua5 suggests that the linker and the SUA5 domain could interfere with enzymatic activity. We set out to test this hypothesis by mutating several conserved residues in the linker and at the domain interface. The sequence conservation score of each residue was calculated using 500 orthologs and mapped onto the Pa-Sua5 structure using Consurf (Supplemental Fig. S6; Ashkenazy et al. 2016). The linker region from Ala\textsuperscript{225} to Ala\textsuperscript{239}, facing the active site, contains several highly conserved residues. Notably the motifs Pro\textsuperscript{228}–Gly\textsuperscript{229}–Met\textsuperscript{230} (Fig. 3C) and His\textsuperscript{234}–Tyr\textsuperscript{235} (Supplemental Fig. S4) are positioned, respectively, near the adenyl-moiety and the phosphate groups of PPI/ATP. To test the importance of these motifs for the activity, we generated the Pa-Sua5\textsuperscript{P228A/G229A/M230A} and Pa-Sua5\textsuperscript{H234A/Y235A} mutants. We also tested the Pa-Sua5\textsuperscript{P237A} and Pa-Sua5\textsuperscript{P237S} mutants, reasoning that this strictly conserved proline could influence the flexibility of the linker region. In addition, we constructed the Pa-Sua5\textsuperscript{R301A/R328A} and Pa-Sua5\textsuperscript{R301D/R328D} mutants to test if the integrity of the domain interface affects the activity of Sua5 proteins.

In the structure of Pa-Sua5, the conserved residues in the linker and SUA5 domain are functionally important in vivo. We used in vivo complementation assays in S. cerevisiae to test if the above-identified residues are functionally important. The absence of the SUA5 gene in yeast results in an extremely slow growth phenotype. Thus, we used as host a S. cerevisiae strain that carried chromosomal deletion of the SUA5 gene and a plasmid Ycplac33 expressing the endogenous SUA5 gene in trans. The genes encoding mutant Pa-Sua5 proteins were then introduced on a second plasmid pESC-LEU. Plasmid shuffling, i.e., selection of the strain which lost the Ycplac33-SUA5 plasmid, was done in restrictive condition by addition of 5-FOA into the growth medium. For all constructs, the presence of the pESC-LEU plasmid and the loss of the Ycplac33-SUA5 plasmid after plasmid shuffling were validated by colony PCR (Supplemental Fig. S8A). In addition, the heterologous expression of all recombinant Pa-Sua5 proteins in yeast cells was confirmed using western blot (Supplemental Fig. S8B).

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Of note, the Suas5\textsuperscript{R301D/R328D} mutant was more affected than the Pa-Sua5\textsuperscript{R301A/R328A} mutant. Expression of the Pa-Sua5\textsuperscript{P228A/G229A/M230A} mutant resulted in an amorphic phenotype (similar to that of Δsua5 strain), indicating that this mutant has no significant activity. Together, the data indicate that the conserved residues in the linker and in the SUA5 domain are functionally important in vivo.

We next measured the in vitro ATPase activity of the Pa-Sua5 mutants corresponding to the amount of AMP detected after 1 h of incubation at 50°C (Fig. 5, black bars). The activities of the Pa-Sua5\textsuperscript{P237A} and Pa-Sua5\textsuperscript{P237S} mutants were similar to that of Pa-Sua5\textsuperscript{wt}. These data are in line with the in vivo data and suggest that the strictly conserved Pro\textsuperscript{237} at the base of the linker is not essential for the function in the conditions tested. Interestingly, Pa-Sua5\textsuperscript{H234A/Y235A}, Pa-Sua5\textsuperscript{R301A/R328A}, and Pa-Sua5\textsuperscript{R301D/R328D}, which partially complemented the yeast growth phenotype, were totally inactive in vitro, suggesting that the activity of these mutants can be rescued in vivo. Combined with the in vivo hypomorphic and amorphic phenotypes of these mutations, this indicates that both the His\textsuperscript{234}–Tyr\textsuperscript{235} motif and the interface residues Arg\textsuperscript{301} and Arg\textsuperscript{328} are important for Pa-Sua5 function. In contrast, Pa-Sua5\textsuperscript{P228A/G229A/M230A} still exhibited significant ATPase activity (an approximate threefold decrease of AMP production as compared to Pa-Sua5\textsuperscript{wt}) even though it did not complement the growth of yeast. This suggests that Pa-Sua5\textsuperscript{P228A/G229A/M230A} does not produce enough TC-AMP to sustain growth or that the
observed in vitro production of AMP does not lead to synthesis of TC-AMP.

Effect of the presence of Pa-KEOS on Pa-Sua5 in vitro ATPase activity

Sua5 proteins together with the KEOPS complex constitute the t6A pathway in Eukarya and Archaea. To test whether KEOPS influences the activity of Sua5, we added in our ATPase assay the Pa-KEOS complex together with cognate substrate Pa-tRNA^{3{\text{Y}}(UUU)}^{\text{tRNA}}$, creating reaction conditions that allow the synthesis of t6A-tRNA. The quantity of AMP produced by Pa-Sua5^{wt} increased approximately twofold in the presence of KEOPS alone or with tRNA but remained unaffected in the presence of BSA (Supplemental Fig. S10). Pa-Sua5^{P237A} and Pa-Sua5^{P228A/G229A/M230A} also showed a small but significant increase of activity ($P$-value < 0.005) (Fig. 5, white bars). Unexpectedly, a significant amount of AMP was detected for the inactive mutants Pa-Sua5^{H234A/Y235A}, Pa-Sua5^{P228A/G229A/M230A}, Pa-Sua5^{R301A/R328A}, and Pa-Sua5^{R301D/R328D} (Fig. 6). This suggests that these mutants hydrolyze ATP but do not form TC-AMP. The mechanisms behind the stimulating effect of KEOPS and its absence in the case of the Pa-Sua5^{P228A/G229A/M230A} mutant remain unknown.

Crystallographic structure of Pa-Sua5^{H234A/Y235A}

The mutant Pa-Sua5^{H234A/Y235A} was co-crystallized with ATP, l-threonine, HCO$_3^-$ and MgCl$_2$ in the same conditions as for Pa-Sua5^{wt}. Crystals belonged also to the C2 space group but with different cell edges from the crystals of the wild-type protein. Data were obtained at a...
We could not detect any in vitro ATPase activity for the SUA5 domain alone. We noticed that the SUA5 domain has no significant sequence conservation, except for residues involved in the interaction with the TsaC-like domain and a motif \( ^{326}\text{MNRLXXA}^{333} \) at the C terminus (Supplemental Fig. S6) found in the a11. Mutations disrupting this interaction in Sua5 from \( P. \text{abyssi} \) provoked a significant loss in activity, indicating that the integrity of this interface is important for enzyme activity.

The linker between the two domains is partly disordered in the St-Sua5 structures while it has a well-defined electron density in our \( Pa \)-Sua5 structures. Notably, the residues from Asp\(^{223} \) to Lys\(^{231} \) form a twisted hairpin that enters the active site and overlaps with the position of AMPPNP or TC-AMP in the St-Sua5 structures (Fig. 3C). This conformation of the linker is also observed in the absence of bound PPI (Supplemental Fig. S3). The linker completely closes the active site, preventing binding of nucleotides (Fig. 3B). We propose that the linker acts as a lid that undergoes conformational changes during the reaction cycle, controlling the access of substrates/products to the active site.

Closure of the active site by surface loops is a recurrent mechanism used by enzymes to create favorable reaction conditions. Although this scenario is plausible for Sua5, it is not clear how TsaC catalyzes the same reaction without a linker and a SUA5 domain. In the case of \( Ec \)-TsaC, it was reported that the last 20 residues are flexible and that their conformation is affected by ATP binding (Teplova et al. 2000; Harris et al. 2015). This prompted Harris and colleagues to hypothesize that the C terminus acts as a flexible arm that may participate in the binding of ATP (Harris et al. 2015). It is therefore tempting to speculate that the C-terminal part of TsaC and the linker of Sua5 play similar roles.

The enzyme TobZ catalyzes the \( O \)-carbamoylation of the antibiotic tobramycin. The structure of TobZ from \( Streptotropha\text{mitis tenebrarius} \) (PDB: 3VEZ) is composed of an N-terminal Kae1/TsaD/Qri7-like domain and a C-terminal TsaC-like domain (Supplemental Fig. S12B; Parthier et al. 2012). Both domains interact closely, creating a wide internal tunnel that connects their respective active sites. The TsaC-like domain catalyzes the formation of carbamoyl-AMP using carbamoyl-phosphate and ATP as substrates. Carbamoyl-phosphate is dephosphorylated and the carbamoyl group performs a nucleophilic attack upon the \( \alpha \)-phosphate of ATP, giving PPI and carbamoyl-AMP. Interestingly, the region between Val\(^{490} \) and Thr\(^{497} \) acts as a gating loop that closes the active site of the TsaC-like domain after ATP binding (Supplemental Fig. S12). Parthier et al. (2012) proposed that the synthesis of carbamoyl-AMP by TobZ proceeds through substrate-assisted catalysis. This hypothesis is motivated by the fact that no clear catalytic side chains are present near the carbamoyl moiety of the substrate. Similarly, TsaC/Sua5 proteins lack obvious catalytic side chains in the substrate binding site, suggesting that they also use substrate-assisted catalysis for the formation of TC-AMP.

**FIGURE 6.** In vitro t\(^6\)A synthesis activity of \( Pa \)-Sua5 proteins. t\(^6\)A activity assays were performed using 2 \( \mu \)M of \( Pa \)-Sua5 and \( Pa \)-KEOPS, 5 \( \mu \)M of \( Pa \)-tRNA\(^{\text{Lys(UUU)}} \), and in the presence of 182 \( \mu \)M L-[\(^{14}\text{C}\)]-threonine. The reaction mixtures were incubated for 1 h at 50° C. The reactions were stopped on ice, and macromolecules were precipitated by addition of 15% TCA. tRNA was recovered on filters, and the amount of radioactivity was determined by scintillation counting. All experiments were done at least in technical triplicates and biological duplicate. The bars correspond to standard deviation. The signal in the negative control sample corresponds to background noise.
After crystallization of Pa-Sua5 with all substrates such that TC-AMP can be formed, only \( \text{L-threonine} \) and PPI were bound to the active site. The formation of TC-AMP is energetically unfavorable (Lauhon 2012); even small concentrations (nM) of PPI present in the crystallization liquid would convert the TC-AMP back to ATP, which may explain why only PPI and \( \text{L-threonine} \) were found in the binding pocket. The binding site of \( \text{L-threonine} \) is deeply buried and not accessible when nucleotide analogs are occupying the active site. Therefore \( \text{L-threonine} \) and \( \text{HCO}_3^- / \text{CO}_2 \) should bind before ATP. In \( \text{Ec-TsaC} \), \( \text{L-threonine} \) also must bind first to allow the correct coordination of ATP in the binding pocket (Harris et al. 2015). In \( \text{Pa-Sua5} \), PPI interacts with the conserved motif His\(^{234}\)–Tyr\(^{235}\) from the linker (Supplemental Fig. S4). Mutation of this motif is important for TC-AMP synthesis, likely by playing a role in the binding of ATP and/or PPI. In our structure, PPI partially overlaps with the position of the \( \beta \)-phosphate moiety of the nucleotide compounds in \( \text{St-Sua5 AMPPNP} \) and TC-AMP complexes, suggesting that PPI may be an inhibitor of Sua5 (Fig. 3C). In agreement with this hypothesis, Lauhon (2012) observed that the TC-AMP synthesis activity of Sua5 from \( \text{Bacillus subtilis} \) is strongly activated in the presence of pyrophosphatase, an enzyme that hydrolyzes PPI into phosphate.

In the current view, the Sua5/TsaC enzymes synthesize an N-carboxy-L-threonine intermediate from \( \text{HCO}_3^- / \text{CO}_2 \) and \( \text{L-threonine} \). Subsequently, N-carboxy-L-threonine attacks the \( \alpha \)-phosphate of ATP to generate TC-AMP and PPI (Lauhon 2012; Perrochia et al. 2013a). However, this putative mechanism has not yet been demonstrated. Unexpectedly, the residual electron density of the \( \text{Pa-Sua5} \)H\(^{1234}\)A/Y\(^{235}\)A mutant revealed the presence of a bicarbonate (\( \text{HCO}_3^- \)) in front of the \( \text{L-threonine} \) (Supplemental Fig. S11). The NH\(_2\) group of \( \text{L-threonine} \) is directed toward the \( \text{HCO}_3^- \) and ion, which likely presents the Michaelis-complex preceding the synthesis of N-carboxy-L-threonine.

The conserved Pro\(^{228}\)–Gly\(^{229}\)–Met\(^{230}\) motif is part of the hairpin that blocks the active site cavity. The \( \text{Pa-Sua5} \)P\(^{228}\)A/G\(^{229}\)A/M\(^{230}\)A mutant hydrolyzes ATP, but it does not complement SUA5 deletion in vivo, nor does it produce \( \text{Pa-Sua5} \)P\(^{228}\)A/G\(^{229}\)A/M\(^{230}\)A in vitro, suggesting it is not capable of TC-AMP synthesis. This mutation could influence the conformation of the linker and may therefore affect the synthesis of N-carboxy-L-threonine and binding of ATP. We therefore propose the following mechanistic model for ATP synthesis by Sua5 proteins. \( \text{L-threonine} \) and \( \text{HCO}_3^- / \text{CO}_2 \) bind first to the narrow cavity at the bottom of the active site. The linker lid subsequently closes the active site favoring the synthesis and stabilization of N-carboxy-L-threonine by excluding solvent and increasing the local concentration of the substrates. The subsequent opening of the lid allows ATP binding followed by nucleophilic attack of the carbonyl group of N-carboxy-L-threonine leading to TC-AMP and PPI.

In conclusion, we identified two novel binding sites in Sua5 proteins: one for the bicarbonate substrate and one for the PPI product. The structure and the functional properties of the linker suggest it plays an active role during the reaction cycle, probably related to the stabilization/synthesis of the N-carboxy-L-threonine intermediate and to the binding of ATP/PPI. The absence of linker and SUA5 domain in the TsaC proteins demonstrates that these proteins use different mechanisms to synthetize TC-AMP. TsaC and Sua5 are therefore closely related but functionally distinct orthologs. The identified functional differences provide clues to help understand the complex evolutionary history of these universal proteins.

**MATERIALS AND METHODS**

**Cloning procedures and mutagenesis**

The recombinant plasmid pET26b(+)–PAB1302–(wt)\(^{651}\) (Perrochia et al. 2013a) was used as template for site-directed mutagenesis. The mutants were constructed using Phusion polymerase (Thermo Fisher). The oligonucleotides used were synthesized (Genewiz) and are listed in Supplemental Table S1. For generating point mutations, the QuickChange Kit II (Thermofisher) protocol was used. For construction of truncated proteins, the sequences encoding for the isolated protein domains were amplified using PCR. A hexahistidine tag or a Strep-tag II was added in 5' positions, the QuickChange Kit II (Thermofisher) protocol was used. The recombinant plasmid pET26b(+)-PAB1302–(wt)\(^{651}\) (Perrochia et al. 2013a) was used as template for site-directed mutagenesis. The recombinant plasmids were introduced into chemocompetent *E. coli* strain TOP10 (Invitrogen). The recombinant plasmid used for heterologous expression of KEOPS complex of *P. abyssi* was previously described (Perrochia et al. 2013a).

**Recombinant gene expression and protein purification**

Recombinant genes were expressed in *E. coli* strains Rosetta2 (DE3) pLysS (Novagen) or XL1 GOLD (Stratagene). Protein overproduction was done in Auto Induction Media Terrific Broth Base including trace elements (Formedium) prepared according to manufacturer’s protocol. Cells were collected by centrifugation, resuspended in lysis buffer 50 mM Tris-HCl pH 8, 200 mM NaCl, 10% glycerol. Lysozyme (0.1 mg/mL final) and anti-protease Roche cComplete ULTRA tablets EDTA-free were added. Cell suspension was homogenized using the high-pressure cell disruptor One Shot (Constant Systems Ltd). After centrifugation at 30,000g for 30 min, the supernatant was heated at 65°C for 30 min, except for the truncated mutants, to precipitate bacterial proteins. Protein precipitate was removed by centrifugation at 30,000g for 60 min. His-tagged proteins from the soluble fraction were purified on a HisTrap HP using an AKTA FPLC system (GE Healthcare). Strep-tagged proteins from the soluble fraction were purified on Strep-Tactin resin (Qiagen). Fractions of interest were pooled and injected on the Superdex 75 HiLoad 16/60 (or 10/300) (GE Healthcare). Fractions containing pure proteins were concentrated, flash-frozen in liquid nitrogen, and stored at -80°C in lysis buffer containing 10%–20% of glycerol. To increase the solubility of Pa-Sua5 truncated mutants we (i) used classical LB media and started induction with 0.5–1 mM IPTG for 1–2 h at OD\(_{600}\) 0.6, (ii) applied a heat-shock at 42°C before induction to induce chaperon
expression, (iii) grew our cultures at 16°C and 22°C, (iv) varied purification buffers between pH 7 and 8.5 with 200–500 mM NaCl and 0%–20% glycerol. To preserve the stability of the purified proteins, storage conditions at 4°C, −20°C, and −80°C in buffer with glycerol between 10% and 30% were tested.

**tRNA substrate production and purification**

The template sequences for in vitro transcription were synthetized and cloned between HindIII and EcoRI restriction sites in pUC18 vector (GeneScript). The gene coding the *Pa*-tRNA<sub>St</sub>-U(U/U) is surrounded by two sequences coding hammerhead ribozyme in 5' and glmS ribozyme followed by a Bsal site in 3' (Price et al. 1995; Batey and Kieft 2007). Plasmids were linearized by the restriction endonuclease Bsal overnight at 37°C. T7 RNA polymerase was purified as previously described (Grodberg and Dunn 1988). Transcription assays contained 80 mM Na-HEPES pH 7.5, 2 mM spermidine, 40 mM DTT, 20 mM MgCl₂, 2.5 mM NTP, 200 µg proteins, storage conditions at 4°C, pH 7,5, 5mM MgCl₂, and 0%–20% glycerol. Eighty microliters of the supernatant with 1 mM glucosamine-6-phosphate for 5 min at room temperature. The reaction was heated at 80°C for 10 min, to inactivate the enzyme, then centrifuged at 10,000 g for 10 min. The supernatant was injected on a MonoQ 5 mL column using an AKTA FPLC system (GE Healthcare). The elution was performed by a gradient of NaCl from 0.3 mM to 1 M in 50 mM Na-HEPES pH 7.5, 5mM MgCl₂. After analysis on a 12% (w/v) polyacrylamide gel containing 8 M urea, the fractions containing the pure tRNA were pooled, ethanol precipitated, and solubilized in 50 mM Na-HEPES pH 7.5, 5mM MgCl₂. The samples were stored at −20°C.

**Differential scanning calorimetry (DSC)**

DSC experiments were performed using a VP-DSC calorimeter (MicroCal). *Pa*-Sua5 proteins were used at a concentration around 13 µM in 50 mM K-HEPES pH 8, and 35 mM KCl. Scanning from 20°C to 100°C at a heating speed of 1°C/min was done for each experiment. Data were analyzed using the MicroCal Origin software provided by the manufacturer.

**Size-exclusion-chromatography coupled with multi-angle laser light scattering**

The molecular weight of the wild-type *Pa*-Sua5 was determined using size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) with the SEC-TDA equipment (Malvern). The Superdex 200 (75) HR 10/300 columns (GE Healthcare) or Bio-SEC3 column (Agilent) for SEC were equilibrated with buffer containing 20 mM Tris–HCl pH 7.5, 200 mM NaCl, and 5 mM 2-β-mercaptoethanol. Eighty microliters of *Pa*-Sua5 at a concentration of 5 mg/ml were injected at a flow rate of 0.5 mL min⁻¹ onto the SEC column and eluted with the equilibration buffer at a flow rate of 0.3 mL min⁻¹. Elution was followed by a UV–visible spectrophotometer, a differential refractometer, a 7° and a 90° angle light scattering detector and a differential pressure viscometer. The OmniSEC software program was used for the acquisition and analysis of the data. BSA was used as standard reference protein of known molecular weight, concentration, refractive index increment (dn/dc = 0.185 mL·g⁻¹), and intrinsic viscosity to calibrate the instrument.

**Crystallization, data acquisition, and structure determination**

*Pa*-Sua5 at a final concentration of 9 mg/mL was either crystallized alone or cocrystallized with ATP/MgCl₂/l-threonine/HCO₃⁻ (all ligands were added at 5 mM) by the sitting-drop vapor diffusion method. Crystals were obtained within 3 d from a crystallization reservoir containing 0.1 M Tris pH 8.5–8.8, 3.5 M ammonium sulfate. Crystals were cryo-protected in crystallization reservoir solution supplemented with 30% glycerol and flash-frozen in liquid nitrogen. The X-ray diffraction data were collected at 100K at the Soleil Synchrotron Light Source on the Proxima 1 beam line (wavelength 0.9801 Å, PILATUS 6M detector). The images were successfully processed and scaled with the XDS package (Kabsch 2010). The data processing and refinement statistics are summarized in Supplemental Table S2. The structure was solved by molecular replacement with the program MOLREP implemented in the CCP4 suite (Winn et al. 2011) using the structure of St-Sua5 as the search model (PDB code: 2EQA). The model building was carried out with the program Coot (Emsley and Cowtan 2004), and the structure refinement was performed with Refmac5 (Winn et al. 2001).

**Sequence alignment and structure comparison**

The sequence conservation was analyzed using the program ConSurf (Ashkenazy et al. 2016) with the structure of *Pa*-Sua5 as query. Based on the default criteria, the program used CS-BLAST to identify the 500 closest homologs by BLOSUM-60 on the Uniref-90 Protein database and aligned them using MAFFT. The conservation rate of each position was calculated with Bayesian algorithms using the 150 homologous sequences with the lowest E-value and mapped on the structure of *Pa*-Sua5. The PDB codes of the structures are 1HRU and 2MX1 for Ec-TsαC, 2EQA, 3AJE, and 4E1B for St-Sua5, 3VEZ for St-TobZ. The structural comparison and the graphic representations of the protein structures were produced with UCSF Chimera (Pettersen et al. 2004). The electrostatic potential representation was rendered and produced using APBS and PDB2PQR (Unni et al. 2011). The ligands’ coordination was analyzed with LigPlot+ (Laskowski and Swindells 2011).

**In vitro assay for the synthesis of t6A-modified tRNA**

The reaction was performed using *Pa*-KEOPS and *Pa*-Sua5 proteins (2 µM each), purified *Pa*-tRNA<sub>St</sub>-U(U/U) (5 µM) in reaction buffer composed of 50 mM K-HEPES pH 8, 35 mM KCl, 10 mM MgCl₂, 1 mM MnCl₂, 5 mM DTT, 1 mM ATP, 10 mM NaHCO₃, supplemented with 182 µM 1-[1-14C]-threonine (0.05 µCi, 55 Ci/m mol) (American Radiolabeled Chemicals). After incubation at 50°C for 1 h, macromolecules were precipitated by addition of 1 mL of Trichloroacetic acid (TCA) 15% and incubated on ice for 1 h. Precipitated material was applied on glass microfiber filters GF/F
(Whatmann), prewet with TCA 15%, using a vacuum apparatus (Millipore). Assay tubes were rinsed with 1 mL of TCA 15%, and filters were washed three times with 1 mL of TCA 5% and three times with 1 mL of ethanol 95%. After extensive drying, filters were placed into scintillation vials and soaked with 3 mL of Emulsifier Safe scintillation cocktail (Zinsser Analytic). Radioactivity was recorded as average counts per minute (CPM) during 5 min, with a Packard Liquid Scintillation Analyzer.

In vitro ATP hydrolysis assay

The ATP hydrolysis reactions (conversion into AMP and PPi) were carried out using Pa-Sua5 (1 or 2 µM) and, when indicated, with Pa-KEOPS (2 µM) and Pa-tRNA58sw(UUU) (5 µM) in the same reaction mixture as for the ΔA assay, supplemented with [α32P]ATP (3 Ci/µmol) (PerkinElmer). The reaction was performed at 50°C and stopped on ice. Radioactive nucleotides were separated by thin layer chromatography (TLC) using 0.4 M KH2PO4 (pH 3.4) as mobile phase. One microliter of the mix was spotted on a 10 × 10 cm PEI-Cellulose plates (Merck), prerun in distilled water. Plates were dried and radioactivity was revealed by phosphorimaging using the scanner Typhoon Trio (GE Healthcare) (Supplemental Fig. S9). Quantification was done with ImageQuantTL software.

For the determination of enzymatic constants, l-threonine and cold ATP were added at different concentrations (3–150 µM and 5–500 µM) to measure the initial velocity of ATP hydrolysis. The enzymes were added to the final concentration of 1 or 2 µM. The reaction was performed with an incubation time up to 6 min for l-threonine titration and up to 8 min for ATP titration. We could not detect any significant background of ATPase activity in the absence of enzymes. The data were then fitted using nonlinear least squares regression applied to the Morrison or Michaelis–Menten equation using Graphpad Prism. Both equations gave very similar catalytic constant values (Supplemental Fig. S5). All the experiments were performed at least in quadruplicates.

Functional complementation assay in S. cerevisiae cells

Yeast cells were grown at 28°C in standard rich medium YEPD (1% yeast extract, 2% peptone, 2% glucose) or selective minimal media minus leucine with 2% galactose (GAL-LEU) or glucose (GLU-LEU) (Sigma or Euromedex). Cells were transformed using the lithium acetate method (Gietz and Schiestl 2007).

For the construction of the YcpLac33-SUA5 plasmid, the wild-type SUA5 gene was amplified by PCR using genomic DNA from strain BY496 as template (see Supplemental Table S1 for oligonucleotide sequences). The insert was cloned into the YcpLac33 vector using Sall/Sacl restriction sites. The resulting construct was introduced in the Δsua5::KanMX strain, derived from the W303 reference strain. The genes encoding wild-type and point mutant Pa-Sua5 proteins were amplified by PCR using the recombinant pET26b(+) plasmids (Cloning Procedures section) as templates. The PCR products were cloned into pESC-LEU vector between BglII/Sacl or BambII/Sall, and the genes were expressed under the control of respectively pGAL10 promoters, producing FLAG-tagged proteins, or pGAL1 promoters, producing myc-tagged proteins. The Δsua5::KanMX–(YcpLac33–SUA5) strain was transformed with the recombinant pESC-LEU vectors and the transformants were selected on GLU-LEU. For each construction, three transformed clones were selected and grown on GLU-LEU. Cells were serially diluted and spotted in parallel on GAL-LEU to induce the plasmid driven expression of Pa-Sua5 variants and on GAL-LEU supplemented with 1 g/L 5-fluoroorotic acid (5-FOA) to counter select YcpLac33–SUA5 plasmid. The plates were incubated at 28°C for the time indicated in the main text.

Coloncy PCR

To validate the genetic constructions, several colonies per clone were selected from the GAL-LEU 5-FOA plates and suspended in 20 to 60 µL NaOH 20 mM. After incubation at 95°C for 45 min and 300 rpm, the extract was centrifuged at 11,000g for 10 min to remove cell debris. Supernatant was diluted and used with the adequate primers for PCR reaction using Phusion (Thermo Fisher). PCR products were sequenced for validation (Eurofins).

Western blot

Protein extraction was done using the standard protocol as previously described (Liger et al. 2011) for FLAG-tagged proteins or the Kushnirov extraction method (Kushnirov 2000) for myc-tagged proteins. Proteins from soluble fractions were resolved by SDS-PAGE on a 15% gel and transferred onto Protran nitrocellulose membrane (Whatman). Detection of myc-tagged proteins was performed using mouse 9E10 anti-myc as primary antibody (1/3000 dilution, Sigma-Aldrich) and goat anti-mouse HRP-conjugated IgG as secondary antibody (1/5000 dilution) (Sigma-Aldrich). Detection of FLAG-tagged proteins was performed using mouse anti-FLAG M2-peroxidase (HRP) (1/10 000 dilution) (Sigma-Aldrich). Immunoblots were revealed using ECL kit SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher).

DATA DEPOSITION

Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data bank under accession numbers 6F89, 6F87, and 6F8Y.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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Structure–function analysis of Sua5 protein reveals novel functional motifs required for the biosynthesis of the universal t^6A tRNA modification


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