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# Mechanism-based strategies for trapping and crystallizing complexes of RNA-modifying enzymes

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## Summary

Post-transcriptional chemical modifications of RNA are maturation steps necessary for their correct functioning in translation during protein synthesis. Various structures of RNA-modifying enzymes complexed with RNA fragments or full-length tRNA have been obtained, mimicking several stages along the catalytic cycle such as initial RNA binding, covalent intermediate formation or RNA product binding. We summarize here the strategies that have been used to trap and crystallize these stable complexes. Absence of the co-substrate transferring the chemical group leads to the Michaelis complex whereas use of a co-substrate analog to a ternary complex. 5-fluoro-pyrimidine containing mini-RNAs have been used as a general means to trap RNA m<sup>5</sup>U methyltransferases covalent complexes and RNA product /pseudouridine synthases complexes. Altogether, these structures have brought key information about enzyme/RNA recognition and highlighted the details of several catalytic steps of the enzymatic reactions.

**Running title:** Crystallization of RNA-modifying enzyme complexes

## Highlights

- Posttranscriptional chemical modification ensures correct functioning of RNAs.

- Several RNA-modifying enzymes structures in complex with RNA are known.
- The enzymatic mechanism has been exploited to crystallize reactions snapshots.
- The complexes structures explain the RNA recognition mode and catalytic mechanism.

## **Introduction**

The post-transcriptional modification of noncoding RNAs generally reinforces their specific tertiary structure and improves their molecular recognition (<http://modomics.genesilico.pl/papers/>)(Agris, 2004). In particular, modifications at positions 34 and 37 of transfer RNAs (tRNAs) play a crucial role in the precise decoding of the genetic code by stabilizing the correct anticodon-codon interactions on the ribosome during protein translation. Other modifications in RNA have a less well-established role but they modulate the structural flexibility of RNA to fine-tune the molecule and regulate various cellular functions (Grosjean, 2005; Motorin and Helm, 2010). The biochemical and structural characterization of the enzymes that catalyze such modifications have mostly focused on enzymes that target ribosomal RNAs (rRNAs) or tRNAs. As a step towards understanding the molecular basis for substrate selectivity of RNA-modifying enzymes and deciphering the catalytic mechanism of the reactions, the crystal structures of several RNA-modifying enzymes in complex with RNA have been solved. tRNA-modifying enzymes have been classified into two groups (Goto-Ito et al., 2009; Grosjean et al., 1996). ‘Group I’ enzymes do not require the L-shape of tRNA whereas ‘group II’ enzymes recognize the three-dimensional structure of the tRNA. The ‘group I’ enzymes is further divided into subgroup Ia, which modify exposed nucleotides located mainly in the anticodon loop and subgroup Ib, which target residues buried inside the tRNA three-dimensional structure.

The crystallization of RNA/enzyme complexes remains a highly challenging task. Apart from several factors generally important for success, such as the length and base content of RNA fragments, homogeneity of RNA and protein preparations (Garber et al., 2002), conditions where the catalytic reaction does not take place and the complex is sufficiently stable have to be investigated. In several cases, and in particular for co-crystallization with RNA fragments, the knowledge of the chemical mechanism of the enzymatic reaction was judiciously exploited to trap stable reaction intermediates. Moreover, for a few enzymes, snapshots of

various intermediates along the catalytic cycle of the reaction have been obtained, shedding light on the dynamics of catalysis (Chimnaronk et al., 2009; Hamma and Ferré-D'Amaré, 2006; Nakanishi et al., 2009; Numata et al., 2006; Zhou and Huang, 2008). To complement the previous reviews on the subject (Ishitani et al., 2008; Iwata-Reuyl, 2008; Li, 2007; Nakanishi and Nureki, 2005), we summarize here the results from the structures of RNA-modifying enzymes complexes, including the more recent ones (Table 1), and focus on the various strategies that have been used for stabilizing and crystallizing these RNA/enzyme complexes at several stages of the catalytic reaction.

### **Use of full-length tRNA for crystallizing the RNA/enzyme complexes**

'Group II' tRNA-modifying enzymes require the tRNA L-shape. These enzymes do not modify RNA fragments, which therefore cannot be used for crystallization to get interesting information on RNA recognition and catalysis. The structures of three 'Group II' enzymes have been solved in complex with a full-length tRNA molecule: lysidine synthetase, pseudouridine synthase TruA and archaeal m<sup>1</sup>G37 methyltransferase (Table 1)(Goto-Ito et al., 2009; Hur and Stroud, 2007; Nakanishi et al., 2009). In addition, several enzymes from Group Ia or Ib have also been crystallized in complex with full-length tRNA (Ishitani et al., 2003; Numata et al., 2006; Seif and Hallberg, 2009; Zhou and Huang, 2008) although they also accept mini-RNA substrates. In all cases, unmodified tRNA transcripts were used.

#### *Low crystallization temperature to slow down the reaction and trap a ternary methyltransferase/tRNA/SAM complex*

Among the enzymes crystallized in complex with a full-length tRNA, several come from thermophilic organisms, such as m<sup>1</sup>G37 methyltransferase from *Methanocaldococcus jannaschii* named aTrm5 (Goto-Ito et al., 2009). The m<sup>1</sup>G37 modification, 3' adjacent to the anticodon, is important in preventing frameshifting during translation. The archaeal enzyme from *Methanocaldococcus jannaschii* that catalyzes the methylation of the N1 nitrogen of G37, aTrm5, was crystallized in complex with tRNA<sup>Leu</sup><sub>UAG</sub> or tRNA<sup>Cys</sup><sub>GCA</sub> and the methyl donor S-adenosyl-L-methionine (SAM). In this case, no special crystallization trick was reported and it is anticipated that, given the enzyme hyperthermophilic activity, the crystallization temperature of 20°C has facilitated the trapping of the RNA/enzyme/SAM complex by slowing down the methyl transfer reaction sufficiently (Goto-Ito et al., 2009). Surprisingly, no crystals were obtained when S-adenosyl-L-homocysteine (SAH) or the SAM



analog sinefungin was used instead of SAM. In both complex crystals, G37 is flipped out into the catalytic pocket and its N1 atom is located 3 Å away from the methyl group of SAM, ready to receive the methyl group. It was proposed that Trm5 specifically selects L-shaped tRNAs as substrates, and thereby acts as a tertiary structure checkpoint in tRNA maturation (Goto-Ito et al., 2009).

*Absence of one of the co-substrates or use of a co-substrate analog.*

All modification reactions, except for the isomerization of uridines catalyzed by pseudouridine synthases, depend on at least a second substrate besides the RNA itself, which transfers the chemical group to the target nucleotide. On the one hand, the structure of the nonreactive binary RNA/enzyme complex is informative about the binding of RNA and the mechanism of specific recognition of the substrate. On the other hand, an analog of the second substrate, which is more easily available than a modified RNA, can be used to obtain the structure of the ternary RNA/enzyme/co-substrate complex that will help to decipher the catalytic reaction itself.

Structural rearrangement of tRNA in the archeosine transglycosylase/tRNA Michaelis complex

Other than aminoacyl-tRNA synthetases, archeosine tRNA-guanine transglycosylase (ArcTGT) was the first tRNA-modifying enzyme, whose structure was solved in complex with full-length tRNA (Ishitani et al., 2003). In archaea, the enzyme replaces guanine at position 15 in the D-loop of tRNA with the archeosine precursor, 7-cyano-7-deazaguanine (preQ<sub>0</sub>). Position 15 is deeply buried inside the tRNA three-dimensional structure and interacts with the variable and T-loops. Therefore, the modification of G15 by archeosine has a crucial role in forming the tRNA core and strengthening the interactions between the T- and D-loops that maintain the tRNA L-shape. In the absence of preQ<sub>0</sub>, the base exchange cannot occur and the enzyme/tRNA complex, which corresponds to the initial Michaelis complex, is stable. The structure of the binary *Pyrococcus horikoshii* ArcTGT/tRNA<sup>Val</sup> complex showed that the enzyme recognizes a tertiary structure of the tRNA completely different from the L-form (the λ-form), in which the D-arm base-pairs are disrupted and the variable loop rearranged and involved in base-pairing interactions. This structure also revealed how the enzyme uses a ruler mechanism to react with the λ-form and precisely position the target nucleotide in the active site by recognizing the phosphate backbone of the acceptor stem (nucleotides 1:72 to 7:66) and that of nucleotides 8 to 13 from the protruded D-arm, and by specifically interacting with the bases of nucleotides 14, 15 and 16 in the catalytic pocket.

### Initial binding of tRNA to lysidine synthetase

In bacteria, tRNA<sup>Ile2</sup> lysidine synthetase TilS modifies cytidine into lysidine at position 34 of the anticodon of tRNA<sup>Ile2(CAU)</sup>, converting the codon specificity from AUG to AUA and preventing the misincorporation of methionine in tRNA<sup>Ile2</sup> during aminoacylation. Interestingly, TilS specifically recognizes and modifies the precursor form of tRNA<sup>Ile2</sup>. This ensures the fidelity of isoleucine codon translation in bacteria because only mature tRNAs are substrates for aminoacyl-tRNA synthetases (Nakanishi et al., 2009). The reaction catalyzed by lysidine synthetase proceeds in two consecutive steps: reaction of the target cytidine and ATP to form pyrophosphate and an adenylated cytidine-tRNA intermediate, which is then attacked by L-lysine to produce AMP and lysidine-containing tRNA. The structure of *Aquifex aeolicus* TilS in complex with L-lysine, ATP and Mg<sup>2+</sup> or in complex with nonhydrolysable AMPPNP revealed the initial binding mode of the enzyme with two of its substrates (Kuratani et al., 2007). Later, the structure of *Geobacillus kaustophilus* TilS in complex with tRNA<sup>Ile2</sup> showed how the enzyme recognizes the entire tRNA scaffold, flips out the target C34, and achieves high specificity for its substrate by interacting with the tRNA acceptor stem and by remodeling the conformation of the anticodon loop (Nakanishi et al., 2009). Structures of the adenylated-tRNA intermediate and of the ternary tRNA/lysine/enzyme complexes are now needed to understand the following steps of the catalytic reaction.

### Snapshots of the thiouridylase-catalyzed reaction

*Escherichia coli* MnmA catalyzes the thiolation of uridine at position 34 (wobble position) in the anticodon of tRNA<sup>Glu</sup>, tRNA<sup>Gln</sup> and tRNA<sup>Lys</sup>. The s<sup>2</sup>U34 modification is essential for discriminating cognate from noncognate codons by stabilizing codon-anticodon interactions during translation. A sulfur-relay system first transfers the sulfur from L-Cysteine to the catalytic Cys199 of MnmA (Figure 1). Before transferring the persulfide sulfur thus formed to the tRNA target uridine, MnmA uses ATP to activate this nucleotide by forming an adenylated intermediate. Three crystal structures of MnmA in complex with tRNA have provided snapshots of the sequential chemical steps of the sulfuration reaction although the sulfur transfer from the relay system to the catalytic cysteine did not take place in these complexes (Numata et al., 2006) (Figure 1). Two forms of the binary MnmA/tRNA<sup>Glu</sup> complex were crystallized in the absence of the second substrate ATP. They represent the initial tRNA-binding state, where the active site adopts an open conformation and U34 is flipped out, and the prereaction state, a closed conformation in which U34 is relocated towards the catalytic pocket due to structural rearrangements of an  $\alpha$ -helix overhanging the active site. Furthermore, an adenylated tRNA intermediate was trapped by co-crystallization

of the binary complex with ATP. Indeed, in the absence of a previous persulfide transfer to the catalytic Cys of MnmA by the sulfur relay system, this ternary complex cannot move forward along the catalytic cycle (Figure 1).

#### Snapshots of the dimethylallyltransferase-catalyzed reaction.

Hypermethylated nucleotides at position 37 of the anticodon of tRNA are important for efficiency and fidelity of protein translation. When the third tRNA anticodon is A, it forms a weak base pair with U at the first codon position in mRNA. The codon-anticodon interaction is then reinforced by the hypermodification of the nucleotide A37, 3'-adjacent to the anticodon, to 2-methylthio-N<sup>6</sup>-isopentenyladenosine (ms<sup>2</sup>i<sup>6</sup>A) (Jenner et al., 2010). Dimethylallyltransferase (DMATase) catalyzes the first step leading to this modification, the transfer of an isopentenyl moiety from dimethylallylpyrophosphate (DMAPP) to A37 in tRNA through a channel. Several structures of the binary DMATase/tRNA complex have revealed the flipped out conformation of the target A37 in the initial tRNA binding state: the *E. coli* enzyme was crystallized in complex with tRNA<sup>Phe</sup> (Chimnaronk et al., 2009; Seif and Hallberg, 2009) and the *Saccharomyces cerevisiae* enzyme in complex with tRNA<sup>Cys</sup>, in the absence and presence of the pyrophosphate product (Zhou and Huang, 2008)(Figure 2). In addition, the structures of the ternary complexes of the *E. coli* and yeast enzymes were obtained by soaking the crystals of the binary complex with dimethylallylthiopyrophosphate, a nonhydrolysable DMAPP substrate analog (Chimnaronk et al., 2009; Zhou and Huang, 2008). Finally, when crystals of the *S. cerevisiae* DMATase-tRNA binary complex were soaked with DMAPP, the transfer reaction occurred within the crystals resulting in a structure in which both reaction products, pyrophosphate and i<sup>6</sup>A37-tRNA, are on their way to leave the enzyme. Altogether, the comparison of these different snapshots of DMATase acting on tRNA gave informations about the dynamics of catalysis (Chimnaronk et al., 2009; Zhou and Huang, 2008) (Figure 2).

#### Snapshots of the pseudouridine synthase TruA-catalyzed reaction

Pseudouridine Ψ, the C5-glycosyl isomer of uridine, is the most common RNA modification and plays an important role in maintaining translational efficiency and accuracy (Agris, 2004). In addition to their catalytic function, Ψ synthases are assumed to act as RNA chaperones, assisting in the folding and structural maturation of their RNA substrates. Six families of pseudouridine synthases that modify site-specifically tRNA and rRNA are known. Representative members of each family are the bacterial enzymes TruA, TruB, RluA, RsuA and TruD, and Pus10 present in archaea and some eukaryotes (Kaya and Ofengand, 2003; Koonin, 1996; McCleverty et al., 2007; Mueller and Ferré-D'Amaré, 2009). These latter

organisms use ribonucleoprotein particles for rRNA pseudouridylation (Hamma and Ferré-D'Amaré, 2010; Kiss et al., 2010). A conserved protein assembly, which includes a catalytic subunit, is associated with a target-specific snoRNA (small nucleolar RNA) guide than can base-pair to rRNA sequences on either side of the target uridine. The transformation of uridine into pseudouridine involves breakage of the glycosidic bond, rotation of the detached base and formation of the C1'-C5 bond. A conserved aspartate in the catalytic site is crucial for catalysis. Two differing mechanisms have been proposed for the cleavage of the glycosidic bond: the nucleophilic aspartate either attacks C6 of the uridine base (Michael addition) or C1' of the uridine ribose (acylal mechanism)(Gu et al., 1999; Huang et al., 1998) (Figure 3). In both cases, the final chemical step is proton abstraction from C5 of the isomerized uridine. Therefore, if uridine is replaced by 5-fluoro-uridine, this step cannot occur and the reaction is stalled at an intermediate stage.

*E. coli* TruA isomerizes uridines at positions 38, 39 and/or 40 in the anticodon stem-loop of tRNAs. The structure of TruA was solved in complex with tRNA<sup>Leu1</sup> and tRNA<sup>Leu3</sup>, in which U39 and U38 are targeted, respectively (Hur and Stroud, 2007). Initially, to prevent catalytic turnover, the D60A mutant enzyme, in which the catalytic Asp60 is substituted by alanine, or tRNA<sup>Leu1</sup> containing a 5-fluoro-uridine at position 39 were used, but no crystals were obtained. The TruA/tRNA complexes that crystallized instead represented three independent stages of the TruA/tRNA reaction: the initial docking complex with the anticodon distal to the active site, an intermediate state where the anticodon is bent toward the active site but where the target base remains stacked, and a reactive conformation where G39 of tRNA<sup>Leu3</sup>, which mimics the target base U39, is flipped out of the stem-loop and oriented toward the catalytic aspartate nucleophile. Comparison of these structures, together with molecular dynamic simulations, revealed that the intrinsic conformational plasticity of the anticodon stem-loop is at the origin of the multi-site specificity of TruA.

### **Use of an RNA fragment for crystallizing the RNA/enzyme complexes**

For crystallizing complexes of rRNA-modifying enzymes, a minimal RNA substrate for co-crystallization has to be identified given the size of the natural rRNA substrate. Moreover, since unmodified tRNA transcripts have unstable tertiary structure compared to the fully modified tRNAs (Derrick and Horowitz, 1993; Hall et al., 1989) and some tRNA-modifying enzymes recognize only a subset of the tRNA secondary structure, these have also been often crystallized in complex with an RNA fragment. This is the case of 'Group Ia' enzymes that

target the anticodon and some ‘Group Ib’ enzymes, which modify the T and D-loops of tRNA. When a mini-RNA has been shown to be substrate of such RNA-modifying enzyme, the corresponding RNA fragment that contains a chemically modified nucleotide at the target position can be used for crystallization to trap an inactive RNA/enzyme complex.

#### *Modification of the C5-position of pyrimidine: use of a 5-fluoro-pyrimidine-containing mini-RNA*

Pseudouridine synthases could operate via two alternate mechanisms that both involve the formation of at least one covalent enzyme/RNA intermediate (Figure 3)(Gu et al., 1999; Huang et al., 1998). C5-pyrimidine-RNA methylating enzymes, such as m<sup>5</sup>U (Kealey et al., 1994) and m<sup>5</sup>C MTases (Liu and Santi, 2000; Walbott et al., 2007) use also a covalent mechanism, in which a nucleophile attacks the C6 position of the pyrimidine to activate the C5 carbon for methylation (Figure 4A). The nucleophile is the conserved aspartate for pseudouridine synthases and a cysteine for m<sup>5</sup>U and m<sup>5</sup>C MTases. According to the mechanism of these reactions, when the C5-hydrogen of the target nucleotide is substituted with fluorine, the reaction is stopped at the proton abstraction step and a stable covalent complex can form. Therefore, 5-fluoro-pyrimidine-containing RNA fragments were used in the crystallization assays to prevent catalytic turnover. It was thought their use would also be a judicious strategy to capture a covalent intermediate along the catalytic pathway.

#### Michaelis complex and product complex of pseudouridine synthases

Apart from TruA, which was crystallized in complex with full-length tRNA, several other pseudouridine synthases were crystallized in complex with an RNA stem-loop, in which the target uridine is substituted by 5-fluoro-uridine (Table 1). These enzymes include *E. coli* and *T. maritima* TruB, which target U55 in the T-loop of tRNA and belong to the subgroup Ib of tRNA-modifying enzymes and several rRNA pseudouridine synthases: *E. coli* RluA, a dual-specificity enzyme responsible for Ψ746 formation in 23S rRNA and Ψ32 in several tRNAs; *E. coli* RluF, a member of the RsuA family that modifies U2604 in a stable RNA stem-loop of 23S rRNA containing a bulge; *Pyrococcus furiosus* Cbf5, the catalytic unit of the ribonucleoprotein pseudouridine synthase. In the X-ray structures, flipping of the target uridine and disruption of the tertiary structure of RNA were observed. However, unexpectedly, even when the formation of SDS-resistant complexes between enzyme and RNA had been demonstrated (Hamilton et al., 2006; Spedalieri and Mueller, 2004), no covalent intermediate was caught in the crystals. In fact, the covalent adduct of TruA with

5FU-containing RNA was shown to be unstable (Gu et al., 1999; Huang et al., 1998; McDonald et al., 2010). Furthermore, analysis on SDS-PAGE gels of dissolved cocrystals of RluA, before and after irradiation, indicated that the covalent adduct is destroyed by X-ray exposition (Hoang et al., 2006).

Instead, the structures of RluF (Alian et al., 2009), TruB (Hoang and Ferré-D'Amaré, 2001; Pan et al., 2003; Phannachet and Huang, 2004), RluA (Hoang et al., 2006) and Cbf5 (Liang et al., 2009) in complex with 5-fluoro-uridine-containing mini-RNA show that 5FU is isomerized to 5-fluoro-6-hydroxy-pseudouridine, the hydration product of **3** in Figure 3A, where X=F. O<sup>18</sup> labeling experiments provided evidence that the presence of the hydroxyl group on C6 originates from direct hydration of the released isomerization product of 5FU rather than from hydrolysis of the rearranged Michael adduct **3** (Hamilton et al., 2006; McDonald et al., 2010; Spedaliere et al., 2004). The possible formation of a stable adduct with 5FU (as in RluA and TruA) was explained by the nucleophilic attack of the catalytic aspartate on the C6 atom of the isomerized 5FU and it was proposed that all pseudouridine synthases rearrange 5FU through a common route (either the Michael addition or acylal mechanism) (Hamilton et al., 2006; McDonald et al., 2010). Nevertheless, the catalytic mechanism is still not completely settled and fine differences between the different enzyme families remain to be investigated.

In addition to the complexes of wild-type pseudouridine synthases, the structure of an inactive mutant of TruB, in which the catalytic aspartate was substituted by asparagine, was crystallized in complex with a 5FU-miniRNA, enabling to visualize an analog of the Michaelis complex, in which the target base was not isomerized (Hoang et al., 2005).

#### Trapping the covalent intermediate of the reaction catalyzed by rRNA methyltransferase RumA.

The structure of RumA, which methylates U1939 within *E. coli* 23S RNA using the SAM cofactor, was determined in complex with a 5FU-containing RNA fragment and SAH. The covalent complex, with the uridine C6 atom attached to the catalytic cysteine, contains both the fluorine and the transferred methyl group at C5 (Figure 4A and B). The target uridine is flipped out and its fluorine faces Glu424, the base that abstracts the C5 proton of the intermediate during the methylation reaction (Figure 4B). The RNA adopts a distorted conformation, where, in addition to the flipped-out target uridine, a second nucleobase is everted and assists in cofactor binding, thereby contributing directly to catalysis.

*Use of a mini-RNA and a mutant enzyme to trap the covalent intermediate of the tRNA methyltransferase TrmA-catalyzed reaction.*

The SAM-dependent methyltransferase TrmA from *E. coli* targets U54 in the T-arm of almost all tRNAs. The m<sup>5</sup>U54 modified base forms a reverse Hoogsteen base pair with m<sup>1</sup>A58, which stabilizes the interaction between the T and D-loops and therefore the three-dimensional structure of tRNA. Since Glu358 is the residue equivalent to the RumA catalytic base Glu424, the E358Q mutant of TrmA was used for co-crystallization with various RNA stem-loops (Alian et al., 2008), which were shown to be substrates of TrmA (Gu and Santi, 1991). Like in RumA, the structure of E358Q-TrmA in complex with an RNA stem-loop and SAH shows the C6 of the target uridine covalently attached to the catalytic thiol group of the Cys324 nucleophile, with a methyl group added in trans to C5 of the covalent intermediate (Figure 4C). Interestingly, in both TrmA and RumA complexes, the RNA loop is refolded into a similar conformation characterized by a nonsequential base stack, substrate selectivity being achieved by flipping out the target uridine and the following two bases into the enzyme active site. Moreover, both proteins use RNA bases (A58 for TrmA and C1941 and C1942 for RumA) rather than a protein residue to stabilize the RNA core after base flipping by occupying the site vacated by the flipped out target nucleotide.

*Use of a substrate analog to trap the Michaelis complex of tRNA adenosine deaminase.*

tRNA adenosine deaminase (TadA) is an essential enzyme that catalyzes the hydrolytic deamination of adenosine to inosine at position 34 of tRNA<sup>Arg2</sup> in prokaryotes (Wolf et al., 2002). TadA from *Staphylococcus aureus* was crystallized in complex with an anticodon stem-loop of tRNA<sup>Arg2</sup>, in which the target adenine was replaced by the adenine analog nebularine (Losey et al., 2006). This choice was based on the mechanism proposed for TadA (Figure 5A), which is derived from that of cytidine deaminases that possess the same active site consensus motif. The attack of the zinc-activated water on the C6 of the target adenine produces a hydrated tetrahedral intermediate that collapses to release ammonia and produce inosine. Nebularine, which has a hydrogen atom in place of the 6-amino group (Figure 5B), cannot undergo the hydrolytic deamination and the noncovalent binary complex is stable. Interestingly, nebularine behaves as a substrate analog rather than an intermediate analog because it was not bound as a hydrate in the active site of TadA.

*Covalent intermediate and product complex of tRNA-guanine transglycosylase.*

Bacterial and eukaryotic tRNA-guanine transglycosylases (TGT) replace guanine at position 34 of four distinct tRNAs by 7-aminomethyl-7-deazaguanine (preQ1), or the hypermodified

base queuine (Q), respectively. PreQ1 is a precursor of Q, the presence of which affects codon selection and prevents stop-codon readthrough and frameshifting (Meier et al., 1985). The mechanism of the TGT-catalyzed reaction (Figure 6A) proceeds by (1) the nucleophilic attack of a catalytic aspartate on the C1' of the target nucleotide G34 to form a covalent enzyme/RNA intermediate and detach guanine, (2) the exchange of the guanine base by PreQ1, (3) the deprotonation of the N9 of PreQ1, enabling a nucleophilic attack on the C'1 of the target G34 sugar to form a covalent link between PreQ1 and the tRNA and lead to the product. The structure of *Zymomonas mobilis* TGT was first determined in complex with preQ1 (Romier et al., 1996). Next, the covalent complex between the enzyme and an RNA stem-loop, which is an effective substrate of TGT (Curnow and Garcia, 1995), was crystallized in the presence of the guanine analog 9-deazaguanine (Xie et al., 2003). Indeed, the use of an analog with a carbon atom replacing the nitrogen at position 9 of guanine, which is unreactive for proton abstraction and glycosidic bond formation, enables trapping the covalent reaction intermediate (Figure 6B) (Correll, 2003). The structure of the ternary complex identified the catalytic Asp residue and revealed that the main structural basis for recognition of the target base comes from an unusual conformation of RNA, in which four nucleotides in the anticodon loop region are flipped out (Xie et al., 2003). Furthermore, crystals of the covalent TGT-RNA-9dzG complex were soaked with PreQ1, leading to the nucleotide replacement, deprotonation of the N9 of PreQ1, glycosidic bond formation and product formation (Figure 6C). The structure of the binary product complex between TGT and preQ1-containing tRNA thus formed provided insight into the conformational changes necessary for the guanine/preQ1 exchange.

### **Formation of RNA duplexes and extension of the RNA helix stem structure in the complexes of RNA fragments with RNA-modifying enzymes**

Although self-complementary small RNAs have the tendency to crystallize in the form of duplexes (Holbrook and Kim, 1997), RNA fragments composed of the tRNA T stem-loop nucleotide sequence form a single stable stem-loop species in solution (Koshlap et al., 1999), and hairpin forms are often found in RNA-modifying enzymes complex crystals (Table 1, Fig. 7). The structure of SAM-dependent *Aquifex aeolicus* KsgA, which catalyzes the dimethylation of residues A1518 and A1519 in 16S rRNA, was obtained in complex with an RNA fragment and with both RNA and SAH (Tu et al., 2009). In these complexes, the RNA stem-loop was not observed. Instead, two RNA molecules form a duplex, in which none of



the mismatched residues in the middle are flipped out, so that these structures do not represent catalytic assemblies of the enzyme. In one crystal form of the *T. maritima* TruB/RNA complex, a duplex fold of RNA was also found: among the three RNA molecules per protein/RNA complex, one adopts a stem-loop structure, whereas the other two form a duplex that stacks end-to-end against the first molecule (Pan et al., 2003). Interestingly, the stem-loop and RNA duplex together mimic the intact tRNA molecule. In this TruB/RNA complex, the RNA makes a large number of crystal contacts and it is thought that the duplex conformation was driven by crystal packing forces. In fact, all reported structures of RNA-modifying enzymes in complex with RNA fragments highlight the importance of nucleic acids contacts for crystal formation. In most cases, the RNA stem structure is extended by coaxial stacking with a symmetry-related RNA molecule or another RNA molecule in the asymmetric unit (Figure 7A to E) (Hoang and Ferré-D'Amaré, 2001; Losey et al., 2006; Phannachet and Huang, 2004). When the target nucleotide is located in the T-loop of tRNA (as it is the case for TruB or TrmA), the extended helix mimics the structure of the stacked T- and acceptor stems of an intact tRNA molecule (Figure 7A and B) (Hoang and Ferré-D'Amaré, 2001). Yet, the crystal contacts between RNA molecules do not always involve co-axial stacking of RNA stems, as observed in the nonphysiologic dimeric arrangement of the RluF/RNA complex, which also provides important crystal contacts although the stem-loops of two adjacent RNA molecules are in interaction with each other through base-pairing (Figure 7F) (Alian et al., 2009). Furthermore, the crystal contacts in the Cbf5 complex are not mediated by the RNA substrate but by two RNA guide molecules (Liang et al., 2009).

## Conclusion

The structures of RNA-modifying enzyme complexes obtained to date have shed light about two important questions: the molecular recognition of the RNA substrates and the mechanism of the enzymatic reactions. The binary RNA/enzyme structures generally helped to understand how a modifying enzyme manages to specifically recognize a single (or a few) RNA substrate(s) from the large pool of RNAs present in the cell and how it selects a single (or several) nucleotide(s) for modification. In fact, the enzymes use common substrate recognition means, such as shape recognition to locate the target site, nucleotide flipping to get access to the active site and sequence-specific contacts with the target base in the binding pocket to initiate the reaction. Formation of the RNA-modifying enzymes/RNA complexes involves conformational changes in the protein, the RNA or both, as observed in other protein-RNA complexes (Williamson, 2000). In addition to the splaying out of nucleotides at

and near the position to be modified, the RNA can adopt a completely new secondary structure upon protein binding (Alian et al., 2009; Alian et al., 2008; Hoang et al., 2006; Lee et al., 2005), the most extensive structural rearrangement of RNA substrate being observed for ArcTGT (Ishitani et al., 2003). Remodeling of the protein active site structure occurs upon binding the cognate RNA and also, occasionally, large domain movements (Chimnaronk et al., 2009; Nakanishi et al., 2009; Pan et al., 2003; Phannachet and Huang, 2004; Seif and Hallberg, 2009). Compared to aminoacyl tRNA synthetases, for which numerous structures of tRNA/enzyme complexes have been determined, the interaction surface between the tRNA and the modifying enzyme is generally less extensive (Numata et al., 2006), which is in agreement with the fact that several tRNA-modifying enzymes (from Class I) can handle a small RNA as substrate. In these latter cases, chemically modified RNA fragment and/or enzyme mutant were judiciously designed to trap stable enzyme-RNA complexes and facilitate their crystallization. For several enzymes, snapshots of the sequential chemical steps have been obtained, providing insights into the reaction mechanism and dynamics of catalysis. If only the binary RNA/enzyme complex has been crystallized, other structures along the catalytic pathway are now needed to complete the catalytic sequence overview.

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**Table 1**

Enzyme	Modified base	class <sup>1</sup>	Reference and PDB code	Co-crystallized RNA	Resolution (Å)	Purification and Crystallization details
<i>M. jannaschii</i> aTrm5	m <sup>1</sup> G37 tRNA	II	(Goto-Ito et al., 2009) 2ZZM, 2ZZN	<i>M. jannaschii</i> tRNA <sup>Leu</sup> , tRNA <sup>Cys</sup>	2.65, 2.95	Co-crystallization
<i>P. horikoshii</i> ArcTGT	preQ <sub>0</sub> 15 tRNA	Ib	(Ishitani et al., 2003) 1J2B	<i>P. horikoshii</i> tRNA <sup>Val</sup>	3.3	Co-crystallization
<i>G. kaustophilus</i> TtlS	k <sup>2</sup> C34 tRNA	II	(Nakanishi et al., 2009) 3A2K	<i>B. subtilis</i> <sup>2</sup> tRNA <sup>Ile2</sup>	3.65	Co-crystallization
<i>E. coli</i> MnmA	s <sup>2</sup> U34 tRNA	Ia	(Numata et al., 2006) 2DER, 2DET, 2DEU	<i>E. coli</i> tRNA <sup>Glu</sup>	3.1, 3.4, 4.4	Co-crystallization
<i>E. coli</i> DMATase (MiaA)	i <sup>6</sup> A37 tRNA	Ia	(Seif and Hallberg, 2009) 3FOZ (Chimnarok et al. 2009) 2ZM5, 2ZXU	<i>E. coli</i> tRNA <sup>Phe</sup> <i>E. coli</i> tRNA <sup>Phe</sup>	2.5 2.5, 2.75	Purification of the noncovalent complex by size exclusion chromatography
<i>S. cerevisiae</i> DMATase	i <sup>6</sup> A37 tRNA	Ia	(Zhou and Huang, 2008) 3EPH, 3EPJ, 3EPK, 3EPL	<i>S. cerevisiae</i> tRNA <sup>Cys</sup>	2.95, 3.1, 3.2, 3.6	Purification of the noncovalent complex by size exclusion chromatography
<i>E. coli</i> TruA	Ψ38, Ψ39, Ψ40 tRNA	II	(Hur and Stroud, 2007) 2NQP, 2NR0, 2NRE	<i>E. coli</i> tRNA <sup>Leu1</sup> , tRNA <sup>Leu3</sup>	3.5, 3.9, 4.0	Co-crystallization
<i>T. maritima</i> TruB	Ψ55 tRNA	Ib	(Pan et al., 2003) 1R3F (Phannachet and Huang, 2004) 1ZE2	17-mer 5FU-mini-RNA 22-mer 5FU-mini-RNA	2.1 3.0	co-crystallization Purification of the covalent complex by hydrophobic chromatography
<i>E. coli</i> TruB	Ψ55 tRNA	Ib	(Hoang and Ferré D'Amaré, 2001) 1K8W	22-mer 5FU-mini-RNA	1.85	Co-crystallization
<i>E. coli</i> D48N-TruB	Ψ55 tRNA	Ib	(Hoang et al., 2005) 1ZL3	22-mer 5FU-mini-RNA	2.8	Co-crystallization
<i>E. coli</i> RluA	Ψ746 23S rRNA, Ψ32 tRNA		(Hoang et al., 2006) 2I82	21-mer 5FU-mini-RNA	2.05	Purification of the covalent complex by anion exchange chromatography
<i>E. coli</i> RluF	Ψ2604 23S rRNA		(Alian et al., 2009) 3DH3	22-mer 5FU-mini-RNA	3.0	Co-crystallization
<i>P. furiosus</i> Cbf5	RNA-guided Ψ rRNA		(Liang et al., 2009) 3HJW, 3HJY	21-mer 5FU-mini-RNA	2.35, 3.65	Co-crystallization
<i>E. coli</i> RumA	m <sup>3</sup> U1939 23S rRNA		(Lee et al., 2005) 2BH2	37-mer 5FU-mini-RNA	2.15	Purification of the covalent complex by anion exchange chromatography
<i>E. coli</i> E358Q-TrmA	m <sup>3</sup> U54 tRNA	Ib	(Alian et al., 2008) 3BT7	19-mer mini-RNA	2.4	Purification of the covalent complex by anion exchange chromatography
<i>S. aureus</i> TadA	I34 tRNA	Ia	(Losey et al., 2006) 2B3J	16-mer nebularine-mini-RNA	2.0	Co-crystallization
<i>Z. mobilis</i> TGT	PreQ <sub>1</sub> 34 tRNA	Ia	(Xie et al., 2003) 1Q2R, 1Q2S	20-mer mini-RNA	2.9, 3.2	Purification of the covalent complex by anion exchange chromatography
<i>E. coli</i> KsgA	m <sup>6</sup> <sub>2</sub> A rRNA		(Tu et al., 2009) 3FTE, 3FTF	duplex of 22-mer mini-RNA	3.0, 2.78	Co-crystallization

<sup>1</sup>tRNA modifying enzymes have been classified in distinct classes depending on whether they require the L-shape structure of tRNA or not. Group Ia enzymes target the anticodon, Group Ib enzymes target positions buried in the L-shape of tRNA, Group II enzymes recognize the L-shape structure of tRNA (Goto-Ito et al., 2009).

<sup>2</sup>The only difference between *G. kaustophilus* and *B. subtilis* tRNA<sup>Ile2</sup> is the base at position 16, which does not contact the protein in the structure.

## Figure legends

**Figure 1.** Snapshots of the thiouridylase reaction catalyzed by MnmA. **A** Initial tRNA-binding state. The target nucleotide U34 (in pink) is flipped out in the active site pocket that adopts an open conformation, the variable segment containing Cys199 (residues 190-211) being folded into a long  $\alpha$ -helix Hv and a residual disordered region. **B** Pre-reaction state. Together with the rearrangement of the variable segment into a  $\beta$ -hairpin-containing loop ( $\beta$ v and Lv) that closes the catalytic pocket, U34 is moved towards the catalytic site by 2 Å to adopt a catalytically productive conformation. **C** Adenylated intermediate state. The catalytic pocket is in the closed conformation. Catalytic Cys102 and Cys199 are activated, as indicated by the breakage of the intramolecular disulfide bond linking them, and the adenylated U34 is shifted further, adopting a more productive conformation. **D** Proposed mechanism for  $s^2$ U34 generation by MnmA. MnmA first activates the C2 atom of U34 by adenylation using ATP. Then, the persulfide sulfur on catalytic Cys199, formed through a sulfur relay system, may directly attack the C2 atom of the adenylated intermediate, which releases AMP. A second possibility (not shown) is that the terminal sulfur of the persulfide is liberated as hydrogen sulfide, which serves as a nucleophile to displace the activated oxygen of the uracil base. In both cases, the sulfur transfer is assisted by Cys102 to break the persulfide linkage, and likely by Asp99 as the acid/base catalyst AH/A<sup>-</sup>.

**Figure 2.** Snapshots of dimethylallyltransferase acting on tRNA.

DMTase is shown as protein surface calculated with the *Pymol* program. Dimethylallylthiopyrophosphate (DMASPP) and the target adenine A37 are colored in yellow and the adjacent nucleotides in green. **A** Yeast DMATase in complex with tRNA<sup>Cys</sup> (PDB code 3EPJ). The target adenine is flipped out and occupies the entrance of one side of the channel. **B** Complex of yeast DMATase with tRNA<sup>Cys</sup> and DMASPP (PDB code 3EPK). DMASPP is located in a hydrophobic pocket in the middle of the channel with its dimethylallyl moiety stacking with the target adenine. The sulfur atom of DMASPP is 3.7 Å away from the N6 of A37. **C** Complex of yeast DMATase with  $i^6$ A37-tRNA<sup>Cys</sup> and PP<sub>i</sub> (PDB code 3EPL). The two binding sites are no longer connected by the channel and both products are on their way out.

**Figure 3.** Two mechanisms of pseudouridine formation.

**A** Michael addition mechanism. **B** Acylal mechanism. Glycosidic bond cleavage occurs by Michael addition of the invariant Asp to C6 of the pyrimidine ring (A) or nucleophilic displacement of the uracil by attack of Asp at C1' of the ribose (B). In both mechanisms,

proton abstraction from C5 is the final step, which cannot take place when the C5 hydrogen is replaced by fluorine.

**Figure 4.** Mechanism of m<sup>5</sup>U MTases.

**A** Michael addition of an invariant catalytic cysteine to C6 of the pyrimidine ring leads to the formation of a covalent methylated intermediate **3**. When X=F, the C5 proton abstraction step cannot occur and a covalent methylated intermediate **3'** is trapped. **B** In the case of RumA, intermediate **3'** was crystallized using a fluorinated mini-RNA substrate. The fluorine atom is indicated in light blue. **C** In the case of TrmA, the intermediate **3** was crystallized by using the mutant E358Q, in which the catalytic base Glu358 that abstracts the C5 proton was inactivated.

**Figure 5.** **A** Deamination mechanism of TadA (Losey et al., 2006). **B** Structure of the substrate analog nebularine.

**Figure 6.** **A** Mechanism of bacterial tRNA-guanine transglycosylase (adapted from (Correll, 2003)). **B** Exchange of G34 by the guanine analog 9-deazaguanine 9dzG catalyzed by TGT traps a covalent RNA/enzyme intermediate with 9dzG occupying the catalytic pocket. **C** When the crystals of the covalent intermediate are soaked with preQ1, 9dzG is exchanged for preQ1, which can undergo deprotonation at N9, leading to the product complex. The protein is shown in green, G34 in black, preQ1 in blue, 9dzG in pink.

**Figure 7.** Crystal packing of RNA-modifying enzymes complexes.

**A** *E. coli* D48N-TruB (PDB code 1ZL3). **B** *E. coli* TrmA (PDB code 3BT7). **C** *E. coli* RluA (PDB code 2I82). **D** *Z. mobilis* TGT (PDB code 1Q2R). **E** *S. aureus* TadA (PDB code 2B3J). **F** *E. coli* RluF (PDB code 3DH3). Enzyme is colored in blue and RNA in pink. One RNA/protein complex is shown in light color, the other in dark color.



Figure 1

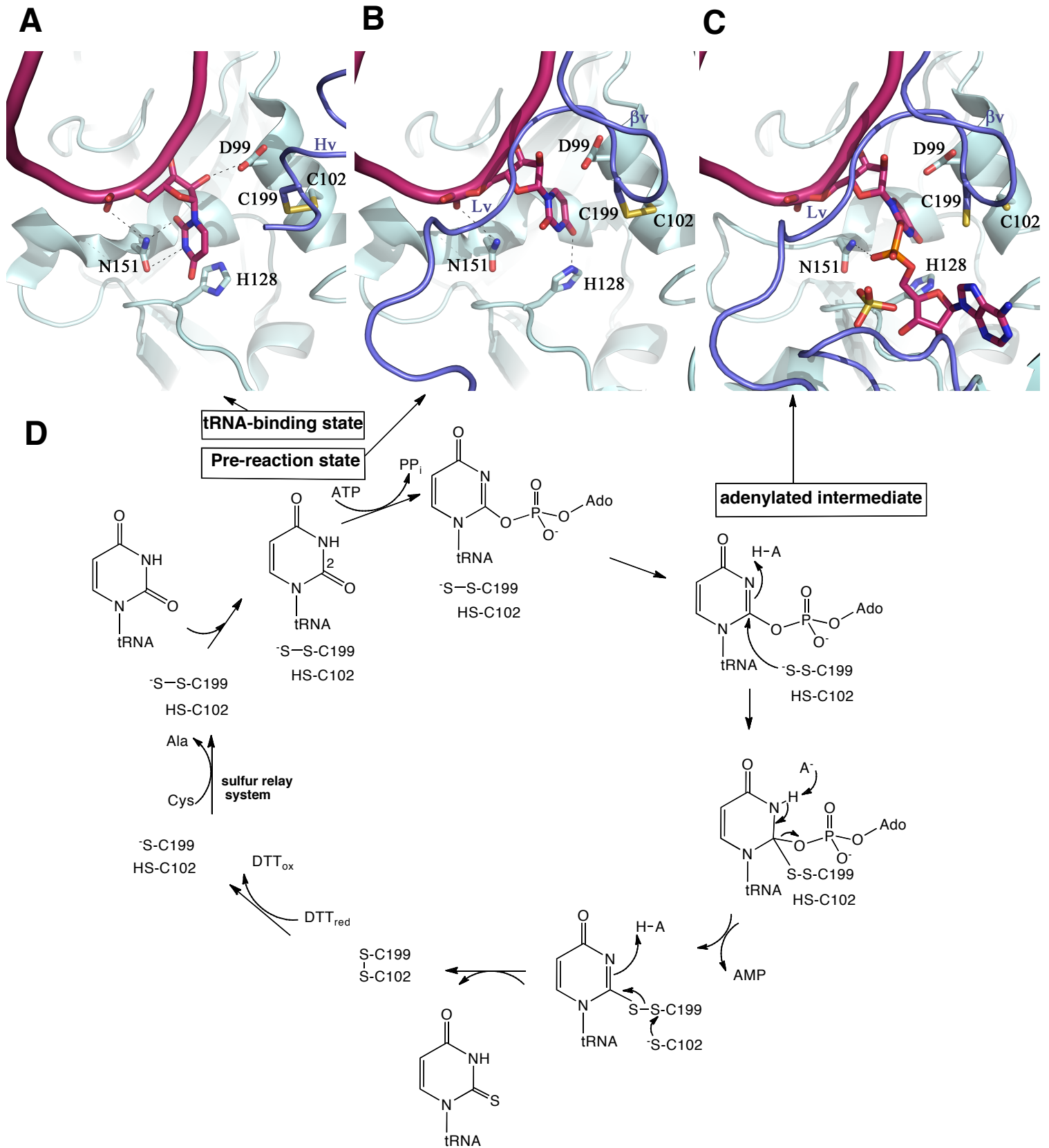


Figure 2

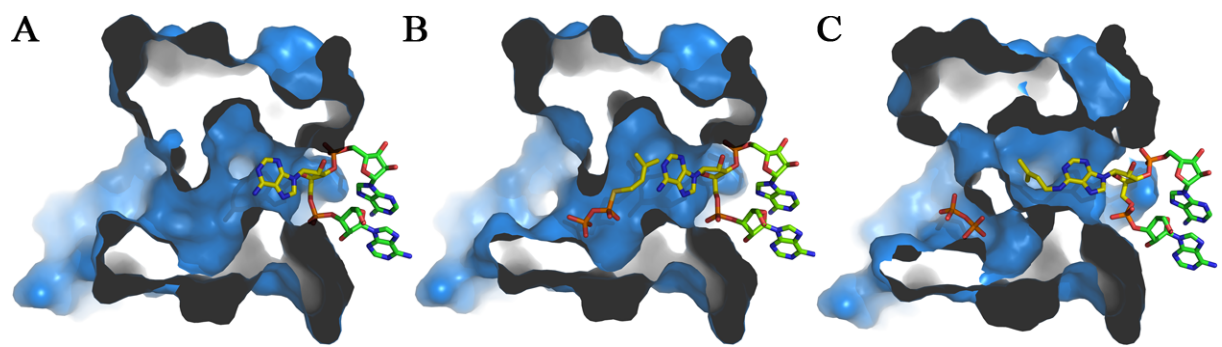


Figure 3

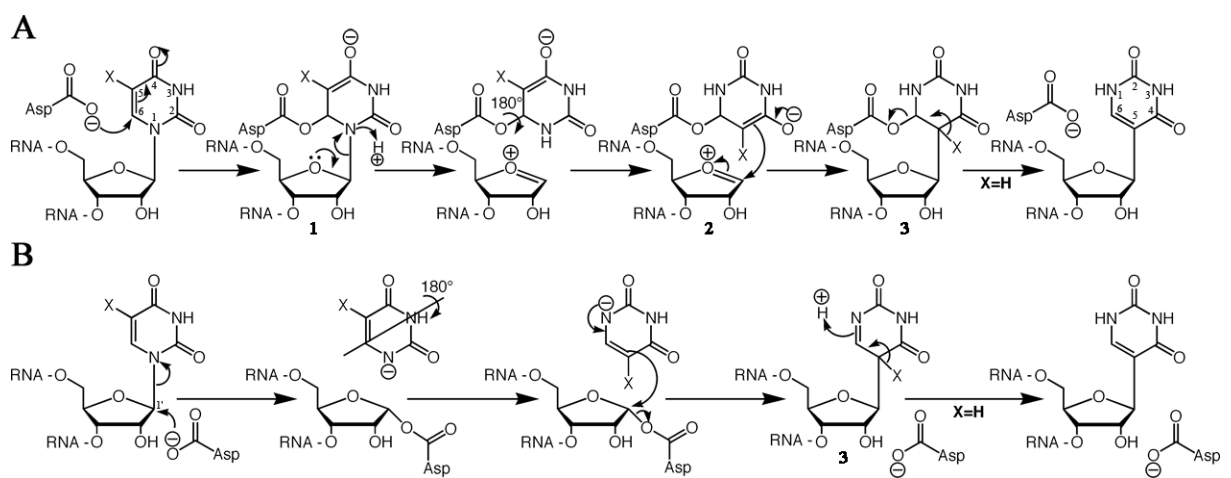
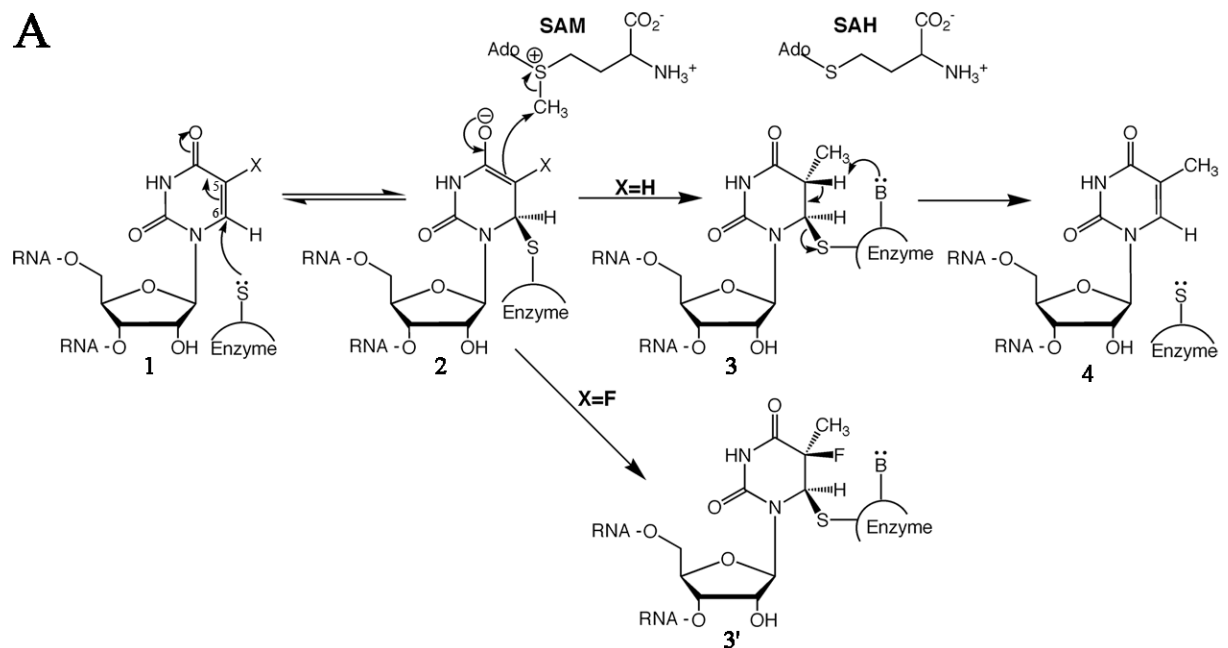
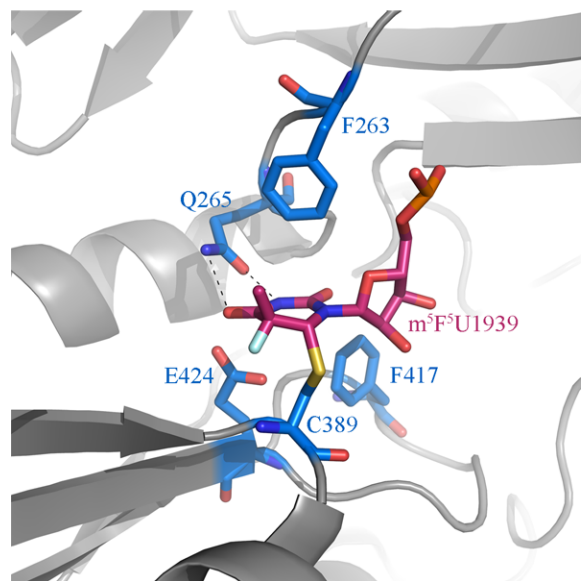


Figure 4

**A**



**B**



**C**

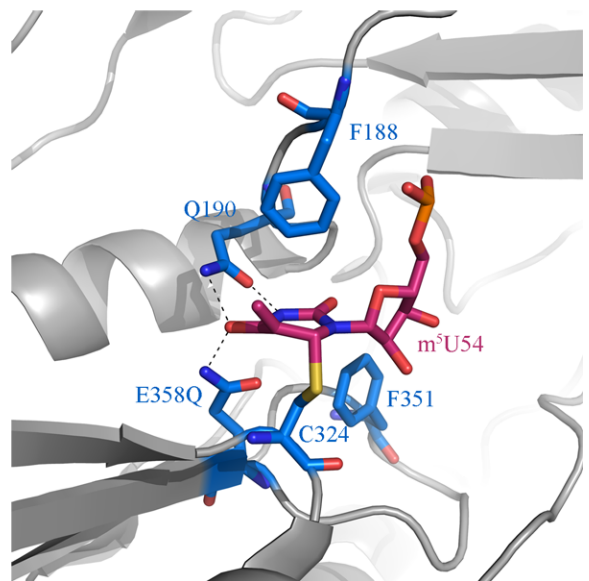
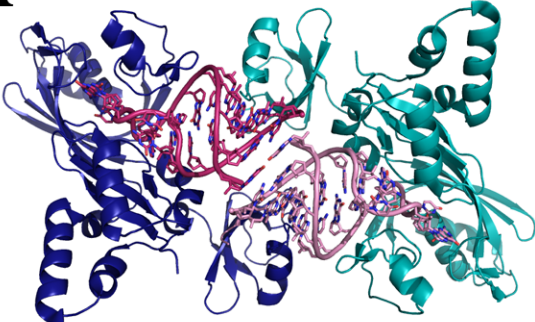


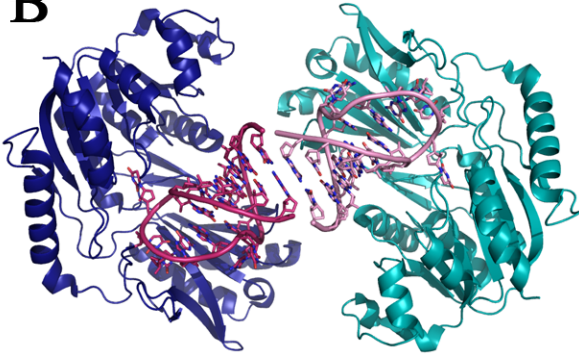


Figure 7

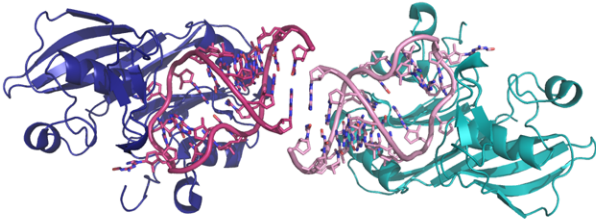
**A**



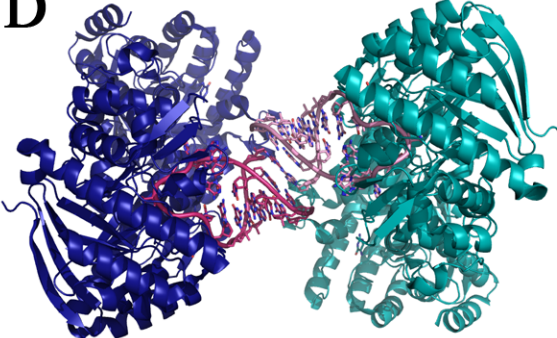
**B**



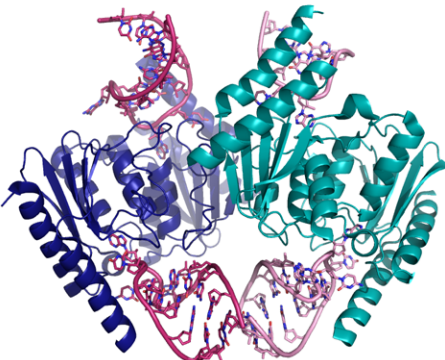
**C**



**D**



**E**



**F**

