Dynamics of RNA modification by a multi-site-specific tRNA methyltransferase

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ABSTRACT

In most organisms, the widely conserved 1-methyladenosine58 (m1A58) tRNA modification is catalyzed by an S-adenosyl-L-methionine (SAM)-dependent, site-specific enzyme TrmI. In archaea, TrmI also methylates the adjacent adenine 57, m1A57 being an obligatory intermediate of 1-methyl-inosine57 formation. To study this multi-site specificity, we used three oligoribonucleotide substrates of Pyrococcus abyssi TrmI (PabTrmI) containing a fluorescent 2-aminopurine (2-AP) at the two target positions and followed the RNA binding kinetics and methylation reactions by stopped-flow and mass spectrometry. PabTrmI did not modify 2-AP but methylated the adjacent target adenine. 2-AP seriously impaired the methylation of A57 but not A58, confirming that PabTrmI methylates efficiently the first adenine of the A57A58A59 sequence. PabTrmI binding provoked a rapid increase of fluorescence, attributed to base unstacking in the environment of 2-AP. Then, a slow decrease was observed only with 2-AP at position 57 and SAM, suggesting that m1A57 formation triggers RNA release. A model of the protein–tRNA complex shows both target adenines in proximity of SAM and emphasizes no major tRNA conformational change except base flipping during the reaction. The solvent accessibility of the SAM pocket is not affected by the tRNA, thereby enabling S-adenosyl-L-homocysteine to be replaced by SAM without prior release of monomethylated tRNA.

INTRODUCTION

Modified nucleosides are abundant and of wide chemical diversity in transfer RNAs (tRNAs). They influence translation accuracy, reading frame maintenance, recognition by aminoacyl-tRNA synthetases and tRNA structure (1–3). The modifications found in RNAs occur post-transcriptionally and range from simple base or ribose methylations to more complex multi-step reactions (4). Most RNA modifying enzymes are mono-site specific, introducing a chemical group at a specific position in a target nucleotide. Nonetheless, there are a few RNA modifying enzymes that display a regional multi-site specificity, modifying consecutive positions in RNA. For instance, Aquifex aeolicus tRNA(N2, N2-guanine)-dimethyltransferase Trm1 catalyzes the methylation not only of guanine 26 but also guanine 27 in tRNA (5,6). In Escherichia coli, the pseudouridine synthase TruA specifically modifies uridines at positions 38, 39 and/or 40 in the anticodon stem loop of tRNAs (7), while rRNA MTase KsgA catalyzes the dimethylation at the N6 position of two adjacent adenines A1518 and A1519 in the small subunit of ribosomal RNA (8,9). Although the multisite specificities of these enzymes have been pointed out, no detailed mechanistic studies have yet been reported. Most of these enzymes have their structures solved but not their complexes with RNA or the RNA was in an unproductive conformation (10). When the structures in complex with RNA are known (7), they reveal the molecular features of the stabilization of the flipped base, but are less informative about the binding, base recognition and flipping processes.

The bi-specific tRNA adenine-N1, A57-A58-methyltransferase (MTase), named TrmI, from the archaeum Pyrococcus abyssi (PabTrmI) methylates two adjacent adenines in tRNA (11). PabTrmI catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to nitrogen 1 of A57 and the adjacent A58 in the
T-loop of tRNAs. In contrast to m1A58, a conserved modification found in all organisms and shown to be essential for cell growth in yeast (12) and for adaptation to high temperatures in thermophilic organisms (13), m1A57 is exclusively encountered in archaea as a precursor of 1-methylinosine (m1I) at position 57 (14,15). In particular, the presence of m1A together with that of m1I was detected after incubation of a tRNAlike transcript with Haloferax volcanii extracts (14), despite the fact that m1A58 has never been found in any of the 51 tRNAs of this species sequenced so far, and these two modified nucleotides were not detected with a transcript in which A57 was mutated to G57. Therefore, it was attested that, in vivo, the formation of m1A57 catalyzed by a SAM-dependent MTase is followed by the deamination of the 6-aminogroup of the adenine moiety by a currently unknown deaminase to produce m1I57.

In general, the multi-site specificity mechanism of these enzymes is not yet well understood. In the case of PabTrmI, we have proposed that the enzyme recognizes the presence of three consecutive adenines (A57A58A59) in P. abyssi tRNAs. Indeed, A58 is methylated by PabTrmI only if an adenine is present at position 59, suggesting that the enzyme modifies the first adenine of an AA sequence (16). Moreover, mass spectrometry (MS) analysis of methylated PabRNA incorporated by PabTrmI indicated the formation of monomethylated A57, in addition to the dimethylated product, but not that of monomethylated A58, suggesting that the enzyme modifies sequentially A57 and then A58. This implies the existence of an RNA binding pocket with a large specificity for the nucleotide to be modified (to accommodate A or m1A) and a rather strict one for the following nucleotide (adenine). Crystal structures of Trmls from several organisms have been determined (17,18) but the structures in complex with RNA are still missing. Yet, the structure of Trml from Thermus thermophilus solved in complex with S-adenosyl-L-homocysteine (SAH) revealed an active site binding pocket suited to bind a flipped-out adenine, suggesting that the enzyme uses a base flipping mechanism (19).

Indeed, RNA modification enzymes commonly introduce such a structural change inside the tRNA to make the nucleoside accessible for modification (20,21). Typically, the enzyme flips the nucleobase to be modified, removing its internal stacking and hydrogen bonding interactions, to expose it to the protein active site.

The most commonly employed method for studying these local nucleotide conformational changes in solution is to replace the target base by 2-aminopurine (2-AP), a fluorescent nucleotide analog (Supplementary Figure S1). The ease, sensitivity and specificity of 2-AP fluorescence detection make the use of 2-AP very attractive. 2-AP is commonly used to probe nucleic acid structure in loops because it rarely affects structure adversely, and its fluorescence is typically enhanced when its environment is disturbed as a result of decreased stacking interactions (22). Therefore, 2-AP is an excellent probe to follow the conformational changes of the target base itself or its neighbors. Indeed, when 2-AP is stacked between DNA bases, its fluorescence is strongly quenched, but the fluorescence increases and shifts when 2-AP adopts a flipped-out position (23,24). This fluorescent analog has also been substituted to nucleobases in strategic positions of single-stranded RNA molecules to act as a probe to monitor folding and unfolding dynamics of RNAs in a few cases (25-28).

We applied this spectroscopic method relying on the 2-AP fluorescence to study the RNA conformational changes occurring during modification by PabTrmI. A57 and A58 are located in the T-loop of tRNA. A58 is involved in a reverse Hoogsteen base pair interaction with U54 within the loop and is more buried inside the tRNA than A57, which is not base-paired. The difference between and 2-AP is the removal of the amino group at position 6 and addition of an amino group at position 2 (Supplementary Figure S1). Thus, 2-AP retains the same base-pairing capacities as adenine but with a different geometry. By replacing A57 or A58 with 2-AP and by following the fluorescence changes during the methylation reaction, we gained deeper insight into the mechanism of PabTrmI. In addition, we built a structural model of PabTrmI in complex with a tRNA, which enabled us to provide a model of the sequence of events along the dual base modification.

MATERIALS AND METHODS

Material

The 2-AP containing RNAs were ordered from Thermo Fisher Scientific. Prior to reaction, they were refolded by heating at 80°C for 5 min, and then cooling on ice for 2 min.

Purification of PabTrmI

The recombinant P. abyssi PAB0283 protein (PabTrmI) bearing a C-terminal His-tag was expressed as described previously (11). After lysis, the cells supernatant was loaded onto a Nickel-NTA affinity column (5 ml; Qiagen) pre-equilibrated in 50 mM Tris-HCl pH 8.5, 500 mM KCl and 200 mM imidazole. The sample was then loaded onto a Superdex 200 HR 10/30 column (GE Healthcare Inc.) equilibrated in Tris-HCl, 500 mM KCl, 200 mM imidazole. The fractions containing the protein were pooled and concentrated with a Vivaspin concentrator (10 kDa PES membrane; Sartorius).

Mass spectrometry analysis

The mini-RNA (20 μM) was incubated for 1 h at 60°C with 10 μM of purified wild-type PabTrmI and 1 mM SAM in 50 μl 50 mM Tris-HCl pH 8, 10 mM MgCl2. The reaction was stopped with 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1) pH 4.5 to precipitate the protein. tRNA in the aqueous phase was extracted by centrifugation at 10 000 g for 5 min, ethanol precipitated and desalted on a MicroSpin G-25 column (GE Healthcare). One microgram of desalted tRNA was then digested at 37°C overnight in 10 μl of 50 mM DHB (2,5-dihydroxybenzoic acid; Sigma-Aldrich, Saint Quentin Fallavier, France) containing 2 μg of RNase A (Fermentas). One microliter of digest was mixed with 9 μl DHB (20 mg/ml in water: methanol 50:50) and 1 μl of the mixture was spotted on the MALDI plate and air-dried.
MALDI-TOF MS and MALDI-TOF/TOF MS/MS analyses were performed directly on the digestion products using a 4800 MALDI TOF/TOF Analyzer mass spectrometer (ABSciex, Les Ulis, France). The instrument is equipped with an Nd:YAG laser (operating at 355 nm wavelength of <500 ps pulse and 200 Hz repetition rate). Acquisitions were performed in positive ion mode except those for determining the methylation kinetics that were measured in negative ion mode to ensure that the positive charge generated on the N1 atom of adenine upon m1A formation does not affect ionization and that the percentage of methylated RNA can be accurately quantified by measuring the area of the peaks corresponding to the non-methylated and methylated fragments. For MS/MS experiments, precursor ions were accelerated at 8 kV and the MS/MS spectra were acquired using 1 kV collision energy with CID gas (argon) at a pressure of 3.5 × 10^-6 Torr. MS data were processed using DataExplorer 4.4 (Applied Biosystems).

Steady-state fluorescence assay

The emission fluorescence spectra of 2-AP containing mini-RNAs alone and in complex with PabTrmI were recorded in a quartz cuvette at 37°C on a Cary eclipse fluorescence spectrophotometer (Varian). The excitation and emission slits were set at 5 and 10 nm, respectively. After exciting at 320 nm, the fluorescence emission was recorded every nm from 335 to 550 nm. The resulting spectra were corrected from the contribution of the buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl2). In all experiments, the concentration of RNA was 0.5 μM while that of PabTrmI was varied. Before recording the spectra, enzyme and RNA were incubated for 5 min to reach the equilibrium.

Stopped-flow kinetics

The experiments were performed on a TGK Scientific SF-61DX2 stopped-flow fluorescence spectrophotometer equipped with a temperature-controlled circulating water bath. RNA (1 μM) was rapidly mixed with PabTrmI (6 μM) alone or in complex with SAM (1 mM) or SAH (1 mM). All reactions were performed in reaction buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl2) at various temperatures. Before rapid mixing, the samples loaded in the stopped flow syringes were incubated for 10 min at the appropriate temperature. 2-AP fluorescence was recorded by exciting at 320 nm and collecting through a WG-360 nm cut-off filter. Up to five transients were collected and averaged for each condition. Background signal was determined against buffer to allow data scaling. The observed rates (k_i) determined by fitting the averaged transients to multiple exponentials equation using the SigmaPlot 12 software. The temperature dependence of each k_i was measured and the energies of activation (E_a) were determined using the following equation: k_i = A*exp(-E_a/RT).

RESULTS

p_{ab}TrmI recognizes and methylates both A57 and A58 in mini-tRNA^{apo} containing the A57A58A59U60 sequence

We have previously shown that p_{ab}TrmI recognizes and methylates the first adenine of an AA sequence in a full-length tRNA (16). While p_{ab}TrmI methylates both A57 and A58 in tRNA^{apo}, it becomes mono-site specific with the P. abyssi A59G tRNA^{apo} mutant, A57 being the only targeted base. To verify if the tertiary tRNA L-shaped structure is required for the multi-site specificity, we tested whether p_{ab}TrmI methylates a 29-mer P. abyssi mini-tRNA^{apo}, which includes the acceptor stem and the T-arm of P. abyssi tRNA^{apo} (Figure 1A). Indeed, several RNA-modification enzymes recognize a stem-loop, in which they can efficiently extrude and modify the targeted base. We used MS to examine if the methylation has occurred and to identify the modified positions. After incubation with the enzyme and SAM, the mini-RNA was digested by RNase A, which cleaves after C and U and generates 3'-phosphate nucleosides, and its fragments were analyzed by MALDI MS (Supplementary Figure S2) (29). When incubated with PabTrmI, the spectrum of digested mini-tRNA^{apo} showed fragments at m/z 1326.15 and 1340.17 (Supplementary Figure S2A) coinciding with the expected masses of mono and dimethylated fragments derived from AAAUp, corresponding to the sequence from A57 to U60 in mini-tRNA^{apo} (Figure 1A). The positions of the modifications at the nucleotide level were then identified by tandem mass spectrometry (MS/MS) analysis (Supplementary Figure S2B). In this technique, individual oligonucleotides of interest, which have been previ-
ously identified by standard MALDI-MS analysis, are isolated and subjected to controlled fragmentation (29). The sequence of the dimethylated fragment obtained after incubation with \( \text{pabTrmI} \) (Supplementary Figure S2B) was determined to be \( \text{m}^1\text{A}_{57}\text{A}_{58}\text{A}_{59} \) sequence. To confirm that \( \text{pabTrmI} \) presence of the \( z_2 \) fragment corresponding to the \( \text{m}^1\text{A}_{57}\text{A}_{58} \) sequence, shows that \( \text{A}_{59} \) is not methylated. Moreover, the presence of the \( z_2 \) fragment corresponding to the \( \text{m}^1\text{A}_{57}\text{A}_{58} \) sequence (\( m/z \) 687.14) confirms that both \( \text{A}_{57} \) and \( \text{A}_{58} \) are methylated.

\( \text{pabTrmI} \) recognizes and methylates only \( \text{A}_{58} \) in mini-tRNA\(^{\text{Asp}} \) (TCT) containing the \( \text{G}_{57}\text{A}_{58}\text{A}_{59}\text{U}_{60} \) sequence. Interestingly, all 43 tRNAs from \( P.\text{abyssi} \) contain either the \( \text{A}_{57}\text{A}_{58}\text{A}_{59} \) or \( \text{G}_{57}\text{A}_{58}\text{A}_{59} \) sequence. To confirm that \( \text{pabTrmI} \) methylates the first adenine of an AA sequence inside the T-loop of tRNA (16), we tested the \( P.\text{abyssi} \) mini-tRNA\(^{\text{Asp}} \) (TCT), which possesses the \( \text{G}_{57}\text{A}_{58}\text{A}_{59}\text{U}_{60} \) sequence (Figure 1D), as a substrate of \( \text{pabTrmI} \). As confirmed by MS and MS/MS analysis (Supplementary Table S1), only \( \text{A}_{58} \) is methylated by \( \text{pabTrmI} \) in mini-tRNA\(^{\text{Asp}} \).

The 2-AP replacing the target adenines in a mini-tRNA substrate is not methylated by \( \text{pabTrmI} \)

The \( P.\text{abyssi} \) mini-tRNAs\(^{\text{Asp}} \) containing 2-AP either at position 57 or 58 (named as \( \text{2AP}_{57} \) and \( \text{2AP}_{58} \)) were incubated with \( \text{pabTrmI} \) and SAM, digested by \( \text{RNase A} \) and the resulting fragments were analyzed by MALDI MS (Supplementary Figure S3). When the adenine at position 57 was replaced by 2-AP (\( \text{2AP}_{57} \); Figure 1B), the mini-RNA was methylated by \( \text{pabTrmI} \) at a unique site, as evidenced by the presence of a + 14 Da fragment (1326.07 Da compared to 1312.21 for the control experiment) (Supplementary Figure S3). Similarly, when the adenine at position 58 of \( \text{mini-tRNA}_{\text{Asp}} \) was replaced by 2-AP (\( \text{2AP}_{58} \); Figure 1C), \( \text{pabTrmI} \) methylated only \( \text{A}_{57} \) (Supplementary Figure S3C and D). However, the methylation of \( \text{A}_{57} \) was not as efficient as that of \( \text{A}_{58} \) in \( \text{2AP}_{57} \), as demonstrated by the large amount of non-methylated fragment.

The methylation of \( \text{A}_{58} \) is not impaired by 2-AP at the adjacent position 57

To further confirm the more efficient methylation of \( \text{A}_{58} \) in a 2-AP-containing mini-RNA substrate, the kinetics of \( \text{m}^1\text{A}_{57} \) and \( \text{m}^1\text{A}_{58} \) formation were followed at 60°C with MS. While \( \text{2AP}_{57} \) was methylated too fast by \( \text{pabTrmI} \) for measuring an accurate rate constant by MS, the time course of methylation of \( \text{2AP}_{58} \) by \( \text{pabTrmI} \) yielded an observed rate constant for methylation of \( \text{A}_{57} \) of 0.05 min\(^{-1} \) (Figure 2). This clearly indicates a more efficient methylation of \( \text{A}_{58} \) than \( \text{A}_{57} \) in the 2-AP-containing mini-RNAs.

Moreover, the methylation kinetics of \( \text{2AP}_{57} \) and \( \text{2AP}_{58} \) by \( \text{pabTrmI} \) are similar to those of the full-length \( P.\text{abyssi} \) tRNA\(^{\text{Asp}} \) (Figure 2).

Interaction of 2-AP-containing mini-RNAs with \( \text{pabTrmI} \)

To know if the interaction of \( \text{pabTrmI} \) with a 2-AP-containing mini-RNA substrate modifies the target base environment, we followed the fluorescence emission spectra from 340 to 460 nm after exciting at 320 nm \( \text{2AP}_{57} \) and \( \text{2AP}_{58} \) in the presence of enzyme (Figure 3). The fluorescence from the protein is negligible under our experimental conditions, the observed variation of fluorescence can be exclusively attributed to a change of the 2-AP environment. In the absence of enzyme, both \( \text{2AP}_{57} \) and \( \text{2AP}_{58} \) fluoresce with a maximum peak at ~373 nm. Addition of a 7-fold molar excess of \( \text{pabTrmI} \) to either \( \text{2AP}_{57} \) or \( \text{2AP}_{58} \) resulted in 91% and 43% fluorescence increase, respectively. This increase of fluorescence is associated with a small blue shift of the maximum peak indicating a polarity change in the 2-AP environment. The 2-AP-mini-RNA fluorescence change with increasing concentrations of \( \text{pabTrmI} \) shows a hyperbolic shape in agreement with a binding event (Figure 3C). The apparent dissociation constants of \( \text{2AP}_{57} \) and \( \text{2AP}_{58} \) for \( \text{pabTrmI} \) deduced from this experiment were 1.7 and 2.8 μM, respectively.
Figure 3. Emission fluorescence spectra of 2AP57 (A) and 2AP58 (B) in the absence (dash lines) and presence of PabTrmI (solid lines). The concentration of RNA was 0.5 μM and that of PabTrmI was 6.5 μM. (C) Fluorescence titration of the 2-AP containing mini-RNAs with PabTrmI. Increasing amounts of enzyme were incubated with 0.5 μM 2-AP-mini-RNA in 50 mM Tris-Cl pH 8.0, 10 mM MgCl₂ at 37°C for 5 min before monitoring the emission fluorescence spectrum (λ<sub>ex</sub> = 320 nm).

Dynamics of RNA binding by PabTrmI in the absence of methyl donor

To further investigate the dynamics of RNA binding to PabTrmI, the pre-steady-state kinetics of 2-AP fluorescence increase at λ<sub>ex</sub> = 320 nm after the rapid mixing of either 2AP57 or 2AP58 with an excess of free enzyme in the absence of SAM were monitored by stopped flow in fluorescence mode. When 1 μM 2AP57 or 2AP58 was mixed with 6 μM PabTrmI, an increase in the fluorescence emission of the 2-AP-containing RNAs was observed within a second (Figure 4), followed by several slower processes. The kinetics of 2AP57 and 2AP58 binding by PabTrmI were best fitted with three exponentials (Table 1). The binding of 2AP57 by PabTrmI was slightly faster than that of 2AP58 (κ₁ and κ₃ of 2AP57 are, respectively, 1.4 and 2.5 times faster than those of 2AP58), in agreement with a better affinity of the enzyme for 2AP57.

Dynamics of RNA binding by PabTrmI in the presence of SAM

To get further insights into the recognition mechanism of A<sub>57</sub> and A<sub>58</sub> under catalytic conditions, PabTrmI was pre-mixed with a saturated concentration of SAM, the methyl donor, before mixing with the 2-AP-mini-RNAs. Importantly, the addition of SAM to the 2-AP-mini-RNAs did not affect the fluorescence level of 2-AP (Supplementary Figure S4). The kinetics were recorded after excitation at 320 nm as aforementioned (Table 2 and Figure 5). Remarkably, not only the shapes of the kinetics curves were different from those observed in the absence of SAM but they also differed markedly between 2AP57 and 2AP58 (Figure 5A and B). In the case of 2AP57, the kinetics were best fitted to three exponentials, a biphasic exponential increase of fluorescence during the first 5 s (7.62 s<sup>−1</sup> and 1.1 s<sup>−1</sup>) being followed by a decrease with a rate constant of 0.12 s<sup>−1</sup> (Table 2 and Figure 5A).

In the case of 2AP58, the kinetics were also triphasic (rate constants of 2.8 s<sup>−1</sup>, 0.6 s<sup>−1</sup> and 0.13 s<sup>−1</sup>) but less complex, with a steady increase of fluorescence until a plateau was...
Table 1. Amplitudes and observed rate constants for the base destacking kinetics of 2AP57, 2AP58 by pabTrmI in the absence of cofactor

|       | A1 (%) | A2 (%) | A3 (%) | k1  | k2 (s⁻¹) | k3  |
|-------|--------|--------|--------|-----|----------|-----|
| 2AP57 | 43     | 39     | 18     | 1.8 | 0.34     | 0.05|
| 2AP58 | 28     | 32     | 40     | 1.3 | 0.2      | 0.02|

Table 2. Amplitudes and observed rate constants for the base destacking kinetics of 2AP57, 2AP58 and G57-2AP58-miniRNA by pabTrmI in the presence of SAM or SAH

|       | A1 (%) | A2 (%) | A3 (%) | k1  | k2 (s⁻¹) | k3  |
|-------|--------|--------|--------|-----|----------|-----|
| 2AP57 + SAM | 36    | 64     | (100)* | 7.62| 1.1      | (0.12)* |
| 2AP57 + SAH  | 22     | 37     | 41     | 2.9 | 0.3      | 0.04 |
| 2AP58 + SAM  | 24     | 48     | 31     | 2.8 | 0.6      | 0.13 |
| 2AP58 + SAH  | 18     | 44     | 38     | 1.3 | 0.2      | 0.04 |
| G57-2AP58 + SAM | 34 | 36 | 30 | 2.5 | 0.3 | 0.04 |
| G57-2AP58 + SAH | 29     | 27     | 44     | 2.5 | 0.3      | 0.03 |

(* this phase represents a decay of fluorescence corresponding to the return of the 2-AP to its canonical position in 2AP57 by pabTrmI. This phase was observed only with 2AP57 in the presence of SAM. The kinetics were measured at 55°C.

reached at ~13 s (Table 2 and Figure 5B). The third kinetics phase was associated with a fluorescence increase, in contrast to 2AP57.

The fluorescence change of 2-AP in G57-2AP58-miniRNA was also monitored under catalytic conditions, in the presence of SAM and enzyme (Figure 5C). pabTrmI did not methylate G57-2AP58-miniRNA (data not shown). Therefore, the use of this mini-RNA, in which the target adenine at position 57 is replaced by another purine that is not modified, enables to set aside the influence of the methylation reaction on the base(s) recognition mechanism. Despite the fact that the presence of the amino group of guanine could have an impact on the kinetics curves, these were very similar between G57-2AP58-mini-RNA and 2AP58. The RNA binding kinetics appeared to be only slightly slower and there was no observed decrease of fluorescence intensity when a guanine is present at position 57 rather than an adenine (Table 2).

Effect of SAH on RNA binding and base recognition by pabTrmI

To investigate the effect of the SAH product on RNA binding, the protein was first incubated with an excess of SAH and then rapidly mixed with the various 2-AP-mini-RNAs in the stopped flow fluorimeter. The major phases of rate constants with 2AP57, 2AP58 or G57-2AP58-mini-RNA in the presence of SAH were very similar (Table 2 and Figure 5). Moreover, these kinetics were also similar to those observed with 2AP58 and G57-2AP58-mini-RNA in the presence of SAM, with no decrease of fluorescence.

Energy associated with the binding of RNA to pabTrmI

To determine the activation energies associated with RNA binding, base recognition and methylation of A57 and A58 by pabTrmI, the kinetics were recorded with 2AP57, 2AP58 or the G57-2AP58-mini-RNA in the presence of SAM at different temperatures (Supplementary Figure S5 and Supplementary Table S2). Raising the temperature enhanced the rate constants with both 2AP57 and 2AP58 without altering significantly the overall shape of the kinetics curves (Supplementary Figure S5). In the case of 2AP57, the overall amplitude of the signal diminished as the temperature increased (Supplementary Figure S5A), indicating a more pronounced temperature dependence for the last phase as evidenced by the larger activation energy ($E_a$ ~27.7 kcal mol⁻¹) (Supplementary Figure S6 and Table S2).

Structural model of pabTrmI in complex with tRNA

We have recently solved the crystal structure of pabTrmI in free form and in complex with either SAM or SAH cofactor (16). Unfortunately and despite numerous attempts, the tRNA/TrmI complex could not be crystallized. To understand the molecular basis for the RNA recognition and binding mode employed by pabTrmI, we built a structural model of the enzyme/tRNA complex (Figure 6). The model was generated by rigid manual docking using the tetrameric structure of pabTrmI in complex with four SAM molecules (PDB code 3MB5) and a full-length matured yeast tRNAPhe (PDB code 1EVV). Each monomer is organized into two domains, the N-terminal (Nt) domain and the catalytic C-terminal (Ct) domain containing the SAM binding pocket. Two pairs of monomers are associated into two dimers that combine to generate a stable tetramer. This oligomeric structure is conserved across evolution, as evidenced by several available crystal structures of pabTrmI orthologs (17). Interestingly, two wide and large positively charged clefts are located on the sides of the tetramer (Figure 6 and Supplementary Figure S7). Two tRNA molecules can be accommodated in these putative RNA binding pockets without clashes. In the model, the T-stem loop and acceptor stem of the tRNAs contact the catalytic domains, the anticodon region being completely oriented outside the protein. In addition, in each RNA binding site, the D-loop of the tRNA interacts with the Nt-domain of one monomer, while the double-stranded acceptor and the T-stems contact the Nt-domain of the second monomer. In this configuration, the T-loop that contains nucleotides 57 and 58 lies in...
Figure 5. Base destacking kinetics of the 2-AP containing mini-RNAs by PabTrmI in the presence of SAM (solid lines) and SAH (dashed lines) measured by stopped-flow spectrofluorimetry at 55°C. PabTrmI (6 μM) and either SAM or SAH (1 mM) were pre-incubated for 10 min in one syringe, whereas the other syringe contained the 2-AP mini-RNA with either SAM or SAH (1 mM). The reaction was started with the rapid mixing of both syringes and the total fluorescence was recorded with a WG-360 cut-off filter after exciting at 320 nm. (A) 2AP57. (B) 2AP58. (C) G57-2AP58 mini-RNA.

Discussion

Our previous results indicated that the bi-specific PabTrmI methylates A58 in tRNAs containing the A57A58A59 sequence only if A57 has already been methylated (16). The present methylation assays indicate that the 29-mer P. abyssi mini-tRNAAsp and mini-tRNAArg constitute good substrates of PabTrmI as they are modified similarly to full-length tRNAs and thus they can be utilized to replace the target adenines by 2-AP. Interestingly, the enzyme did not methylate 2-AP, thereby discriminating between adenine, which contains an amino group at the C6 position, and 2-AP, in which this group is present at C2 instead of C6. Methylation kinetics under single-turnover conditions showed that A58 is more efficiently methylated than A57 when 2-AP is present at the adjacent position (57 and 58, respectively). The enzyme methylates quite efficiently A58 in 2AP57 probably because the introduction of 2-AP at position 57 does not much interfere with the T-loop structure nor the correct placement of A58 in the active site. In contrast, in 2AP58, the less efficient methylation of A57 may be due to the non-native reverse Hoogsteen base pair between U54 and 2-AP58 and/or steric hindrance caused by the NH2 group at C2, which could hamper the optimal positioning of the nucleotides. PabTrmI also methylated efficiently A58 in a mini-RNA containing a guanine at position 57. Hence, methylation occurs at position 58 when position 57 contains a methylated adenine, an adenine derivative or guanine, whereas the methylation at position 57 strictly requires adenine 58 to proceed efficiently. This supports our previous conclusion that A57 is methylated before A58 in tRNAs containing the A57A58A59 sequence (16).

The fluorescence properties of 2-AP enabled us to study the binding of RNA to PabTrmI. The observed increase of the 2-AP fluorescence suggests that the binding of PabTrmI to the 2AP-containing RNAs provokes base unstacking of the fluorescent probe itself and/or its immediate neighbors. Interestingly, this variation of fluorescence is in the same order of magnitude as that measured for placement of the target base in the active site of two RNA modifying enzymes (30–32). Accordingly, the model of PabTrmI in complex with tRNA clearly underlines that the enzyme has to employ a base flipping mechanism to get access to both A57 and A58. The flipping of several bases in the vicinity of the target base by RNA modifying enzymes is a widely used mechanism, as reviewed by Li (21). The calculated appar-
ent $K_d$ values for 2AP57 and 2AP58 of 1.7 and 2.8 μM, respectively, indicate that both 2-AP-mini-RNAs are recognized by $p_{ab}$TrmI. Although the enzyme exhibits a slightly higher affinity for 2AP57, in agreement with the slightly faster binding of 2AP57 by $p_{ab}$TrmI compared to 2AP58, the position of 2-AP does not substantially alter RNA binding.

The binding kinetics of the 2-AP-containing mini-RNAs were then analyzed by stopped flow in the presence or absence of SAM or SAH. The rather complex multiphasic kinetics indicate that several molecular changes around the target bases occur, when $p_{ab}$TrmI binds to the RNAs. Similar complex kinetics have been observed for adenine glycosylase MutY (33). Indeed, stopped-flow fluorescence analysis of MutY, using a duplex oligodeoxyribonucleotide containing a uracil-2-AP base pair, revealed that the extrusion of the targeted base was followed by slower protein isomerization steps that provided an additional stabilization of the flipped out adenine in the active site, thereby facilitating excision and base repair.

In the absence of SAM, the fluorescence increase was not followed by any decrease, suggesting that $p_{ab}$TrmI recognizes both target bases in 2AP57 and 2AP58 and that 2-AP (and/or its neighbor nucleotides) may be stabilized in a destacked conformation. The enzyme is therefore able to recognize and bind RNA even in the absence of methyl donor. Remarkably, in the presence of SAM, a decrease of fluorescence occurred after the initial fluorescence increase only in the case of 2AP57. The first two increasing phases represent most likely the series of molecular events regarding the binding of the RNA and base recognition, while the last decreasing phase may be attributed to RNA dissociation after methylation of A58. The absence of fluorescence decrease with 2AP58 could reflect the fact that 2-AP remains bound to the enzyme catalytic pocket or the return of 2-AP in the canonical position inside the tRNA is an extremely slow process. The similar kinetic constants shared by 2AP58 and G57-2PA58-mini-RNA, which does not undergo methylation, indicate that the observed signal of fluorescence likely originates from common events, i.e. RNA binding and base recognition in the vicinity of 2-AP. The kinetic curves for these mini-RNAs, compared with those for 2AP57, also suggest that the environment changes occurring around the 2-AP region are maintained as long as the methylation at position 58 is not achieved. Indeed, the methylation of A58 provides a gain in the stabilization energy of the U54-A58 or T54-A58 base-pairs of 6.8 kcal/mol (34), which agrees with the idea that methylation of A58

Figure 6. Model of $p_{ab}$TrmI in complex with tRNA. (A) Cartoon, (B) surface and (C) electrostatic representations of the enzyme/tRNA complex oriented in the same fashion. The different secondary structures of the tRNA are colored as follows: the T-psi stem loop in pale green, the D-stem loop in cyan, the anticodon stem loop in red, the variable loop in blue and the acceptor stem in salmon. The Nt and Ct domains of $p_{ab}$TrmI are in violet and green, respectively. The SAM cofactor and the target adenines are represented in red. (D) 90° rotation view of (C).
Although the subEnbTrmI tetramer binds four SAM molecules, our model of its complex with tRNA underscores the symmetric binding of two tRNA molecules and the use of only two SAM molecules over four for catalysis. The binding of two tRNA molecules is in agreement with the MS experiments carried out with subEnbTrmI (19). Our model shows that the protein interacts mainly with the T-psi stem loop and the acceptor stem of the tRNA, which is in agreement with our activity test showing that a mini-RNA substrate containing only these regions is recognized by subEnbTrmI and methylated. The model also illustrates that the flipping of both A57 and A58 is mandatory to bring both targets at a suitable distance for methyl transfer. In particular, flexible loops of the Nt-domain that are in close contact with the T-loop could be determinant to untighten the tertiary interactions at the elbow of the tRNA (intersecting region between the D- and T-loops) and promote base flipping. Except base flipping, no dramatic conformational change of the tRNA appears necessary for the reaction to occur, as observed for most RNA-modifying enzymes. In particular, T. thermophilus dihydrouridine synthase, which modifies U20 in the D-loop of tRNA, does not require extensive conformational changes in the tRNA to access its target base because the canonical D-loop/T-loop interaction was maintained in the crystal structure of the complex (35). The larger RNA binding pocket of subEnbTrmI compared to that of other mono-site-specific TrmIs (17) may enable much more flexibility in the binding of the tRNA and would allow the enzyme to position A57 or A58 for methylation without serious steric hindrance. In addition, the rather large solvent accessibility of the SAM binding pocket, even in the presence of tRNA, agrees with the fact that the first SAH molecule could dissociate after methylation of the first adenine and be replaced by the second SAM molecule without provoking tRNA dissociation, thereby enabling the consecutive methylation of the second adenine. This scenario is supported by the comparison of the crystal structures of subEnbTrmI alone, in complex with SAH or SAM, which does not highlight noticeable conformational changes (16). A general mechanism for the double methylation of tRNA by subEnbTrmI can thus be proposed (Figure 7).

Multi-site RNA modifying enzymes may share a similar sequential dynamical process and the same method that uses oligonucleotides containing 2-AP at the target positions may be implemented for the investigation of the RNA conformational changes occurring during catalysis by these enzymes.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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